level had risen from 32 per cent to the 60 per cent level. The rise continued, and a level of 91 per cent was reached on the 9th day of treatment. This abrupt 9 day rise should be contrasted with the slow rise (4 months) shown in Chart 1 when bile alone is fed. The slow rise in the latter case indicates that our stock diet when mixed with bile yields but small amounts of the vitamin. This sluggish rise with bile alone is also in accord with our human cases (8, 13), which indicate a recovery period of weeks with bile alone in contrast to a recovery period of days when the bile is supplemented with the vitamin. These findings differ somewhat from those of Greaves and Schmidt (7). They state that in the bile fistula rat the plasma prothrombin shows a marked rise after the brief period of 2 to 4 days of simple bile feeding. One must assume either that their diet contains far more naturally occurring vitamin than in our experiments, or that a small animal like the rat has proportionately smaller vitamin requirements or has a strikingly shorter recovery period. Then, too, one must consider the different technique for the determination of prothrombin. Our method is a two-stage titration procedure which permits complete control of all variables.

Dog 4, at the 22 per cent prothrombin level, shows an initial 7 day period of treatment with large quantities of the vitamin, but without adding bile to aid in its utilization. There appeared for several days to be a slight response, but this gain was lost, suggesting that the entire fluctuation may not have been related to the change in diet. At any rate, the vitamin, administered alone, has little curative effect. The second experimental period, shown several days later, involved feeding a similar quantity of the vitamin, along with bile salt. It is evident that the bile salt enables the animal to use the vitamin supplement, for within the brief period of 7 days the prothrombin rose from the 25 per cent to the 66 per cent level.

Dog 5, Chart 2, was given a brief period of treatment with carotene and vitamin D. This experiment was prompted by the reports of McNealy, Shapiro, and Melnick (14) and Boys (15) that jaundiced human bleeders are to some extent relieved of their bleeding tendency by adding vitamin D to the diet. In accord with their recommendation, we included a bile salt supplement to aid absorption. The results, however, were very disappointing, for the plasma prothrombin

values remained constant within the limits of experimental error. That this dog was capable of reacting promptly to vitamin K supplements was shown in the second experimental period indicated in the chart. With vitamin K concentrate plus bile salt the prothrombin rose in 6 days from the 40 per cent to the 90 per cent level.

It will be noted that in both dog 4 and in dog 5 we used bile salt in place of whole bile. Under these conditions the bile salt appears to be highly effective, indicating that all the necessary factors for prothrombin manufacture are present. The incomplete recovery of dog 2 (Table I) on bile salt alone may merely imply that difficulty is encountered when the vitamin is present in minimal quantities, as in the stock diet given to that dog.

The rapid rise in prothrombin levels shown in Chart 2 ceased in all cases as soon as the dietary supplement was discontinued. Evidently the vitamin was used promptly, and little or none was stored. This is also shown by the fall in prothrombin which commenced as soon as the vitamin concentrate was withheld. Data, not given on this chart, show that this fall continues steadily, reaching the former low levels in several weeks.

DISCUSSION

Our earlier experiments with fistula dogs, like those of Hawkins and Whipple, indicated that prothrombin reaches the bleeding level in 3 or 4 months. Human cases with bile excluded from the intestine often continue for even longer periods without hemorrhages, though at times they bleed after a few weeks of biliary obstruction. This tendency to earlier bleeding in human adults is in good part due, we believe, to the fact that in man the danger zone is at a higher prothrombin level than in dogs. In some of our more recent experiments on dogs the fall in prothrombin was unaccountably slow in making its appearance. Several dogs were still above the 45 per cent level at the end of 6 months. In these cases the feces remained free of bile pigment, which excluded the possibility that the biliary tract had spontaneously reformed. To rule out the possibility that the kennel diet had varied in its vitamin content we substituted diets containing restricted amounts of vitamin K (diets 9 and 31). Even then, the prothrombin remained for long periods at about the 50 per cent level.

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This extreme variability among dogs leads us to conclude that unknown factors modify the rate at which the prothrombin level falls. One might postulate the existence of large vitamin K reserves in such animals. Again, it is thought that in chicks the vitamin is manufactured by bacteria in the bowel, though mainly in the lower portion of the bowel where it may be poorly absorbed. Bacterial synthesis can be assumed to occur in mammals also, and a variable amount of absorption may occur. The factor of resorption is difficult to control. We have attempted, however, to minimize coprophagy in several of our dogs, but without manifest influence on the rate at which the prothrombin fell.

The mechanism by which vitamin K aids in the manufacture of prothrombin is still obscure. We have supplied evidence to the effect that the liver is vitally concerned in the manufacturing process (16). Among the various possible rôles one must consider the possibility that it is an important building stone for prothrombin, or that it is needed for proper function of the liver cell.

We have not as yet determined in fistula dogs the minimal effective dosage of vitamin K. It may well be that maximal prothrombin response would be obtained with much smaller amounts than we have used. Chick studies have shown that the vitamin is fairly widely distributed in nature, and there can be no doubt that our standard mixed diets do contain the vitamin in moderate amounts. This diet supplemented with bile gives a very slow response. It thus seems likely that the vitamin dosage added by way of supplement is not enormously in excess of optimal requirements. This is a matter which requires further study both in fistula animals and in human jaundiced bleeders. Standardization studies must also include a critical survey of the chick assay technique, for obviously the dosage employed in both man and animals should be based upon accurate assay of the vitamin concentrates employed.

SUMMARY

In biliary fistula dogs the plasma prothrombin falls eventually to low levels and bleeding commonly occurs. Faulty absorption of vitamin K from the intestine in these animals is an important causative factor. Feeding bile permits absorption of the traces of this

vitamin normally present in mixed diets, and as a result a slow rise in prothrombin level is observed. If a standard diet is supplemented with large amounts of vitamin K concentrate the prothrombin rise is rapid, provided bile or bile salt is supplied to aid in the absorption.

Variations in the rate of prothrombin depletion in biliary fistula dogs kept on constant diet indicate the existence of additional factors which require further study. Our experience indicates that vitamin A and vitamin D supplements do not correct the prothrombin deficiency in biliary fistula animals.

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A MENINGO-ENCEPHALITIS IN CHICKS PRODUCED BY THE INTRACEREBRAL INJECTION OF FOWL POX VIRUS

By G. JOHN BUDDINGH, M.D.

(*From the Department of Pathology, Vanderbilt University Medical School, Nashville*)

PLATES 40 AND 41

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The lesions of fowl pox are readily produced experimentally by the inunction of infectious material into the scarified or slightly injured epithelium of susceptible fowls (1). The characteristic eruption which closely resembles that of the natural disease will then appear at the site of inoculation in the course of 3 or 4 days. The virus is found to be present in greatest abundance in the local lesions. It has a specific predilection for multiplying within the susceptible epithelial cells which at the sites of infection become hyperplastic and hypertrophied, followed in the later stages of the disease by necrosis and ulceration.

The presence of the virus within the susceptible epithelium is morphologically recognizable in the pathognomonic intracytoplasmic inclusions. These structures have been shown by Woodruff and Goodpasture (2) to be composed of a mass of smaller elementary or Borrel bodies which are surrounded by a lipoid capsule. The elementary bodies are now generally considered to represent the actual virus.

A generalized distribution of the virus throughout the organism of the infected host seems to take place during the course and long after recovery from the spontaneous as well as the natural disease. Inoculation of the blood or suspensions of the various organs and tissues of infected fowls into the skin of other susceptible birds has demonstrated this fact (3–5). In spite of the widespread and prolonged dissemination, actual proliferation of the virus, as indicated by the presence of the specific inclusion bodies, seems to take place only within the epithelium of the skin and the mucous membranes of the upper respiratory tract and conjunctivae.

The specific affinity of the virus for epithelial cells can be demonstrated by intravenous injection of infectious material. A typical eruption will then develop at the sites of predilection for the natural disease, namely the comb and wattles, angles of the beak, conjunctivae and the upper respiratory tract. If, following this method of inoculation, the skin in any particular area is injured by scarification or plucking the feathers the eruption will appear at this site (6). This specific affinity of the virus of fowl pox for epithelial cells, or in other words its epitheliotropism, is perhaps its most distinct characteristic. This affinity is

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apparently not readily altered in the course of the natural or experimental infection. In the natural infection the portal of entry for the virus is presumably the mouth or upper respiratory tract, situations in which the virus is immediately introduced into its natural environment, the epithelial cells. By the usual experimental inoculations the same conditions are more or less closely repeated.

Introduction into a foreign environment, particularly by intracerebral inoculation into susceptible animals, may markedly alter the behavior of certain viruses. Thus Theiler (7) was the first to show that by repeated intracerebral passage in mice the virus of yellow fever was greatly enhanced in its neurotropic potentialities. A "fixed" virus was thus obtained which on intracerebral inoculation caused the death of the majority of mice within 4 or 5 days after inoculation. It has further been shown by the work of numerous other investigators that the fully fixed neurotropic virus differs from the original pantropic virus in that its affinities for nervous tissues are greatly enhanced while that for other tissues, particularly its hepatotropism, is greatly reduced.

The behavior of vaccinia virus can also be greatly changed by intracerebral passage in rabbits as shown by Levaditi and Nicolau (8). After testicular passage in the rabbit the virus was by repeated intracerebral passage completely adapted to this environment. This neurovaccine was shown by Douglas, Smith and Price (9) to be greatly increased in its virulence as compared with dermovaccine for the skin and viscera of rabbits. In a comparative analysis of the lesions produced by neurovaccine and dermovaccine in the chorio-allantoic membrane of chick embryos (Buddingh, 10), the former was distinguished by an increased capacity for infecting cells of mesodermal origin as well as by a marked increase in virulence for ectodermal epithelium.

Levaditi and Nicolau (8) undertook the intracerebral inoculation of fowl pox virus into several adult hens. The lesion which developed was described as being closely analogous to that produced in rabbits by the intracerebral inoculation of vaccinia virus. Perivascular proliferation, infiltration and parenchymatous alterations were found. They did not report the presence of intracytoplasmic inclusions in the cells in and around these lesions. Lipschutz (4) also reported similar attempts with pigeons without definite results.

The following report will be concerned with our experience and observations on the disease and pathological lesions produced in young chicks by the intracerebral inoculation of fowl pox virus.

EXPERIMENTAL

The strain of fowl pox virus used in these experiments was one which had been maintained in this laboratory for many years by occasional transfer in the skin of chicks. Between transfers it was stored in a dried state over calcium chloride.

A short time before undertaking this experiment this strain of virus was propagated in the chorio-allantoic membrane of chick embryos in pure culture through

All procedures have been carried out under controlled sterile conditions.

1. Ultrafiltration.—The ultrafilter, which was employed in all of the experiments described below, supported a membrane 4 cm. in diameter placed above a Whatman No. 1 filter paper on a perforated metal plate. A thick walled glass cylinder was clamped, by means of a metal cap, onto a rubber washer placed above the membrane. An outlet tube from the cap permitted positive pressure to be applied from a nitrogen tank, whenever necessary. The capacity of this filter was 75 cc. It was set up in a 500 cc. suction flask.

The membranes used were the commercially available Zsigmondy ultrafiltration filters,[1] made from cellulose esters by slow evaporation of their organosols to thin gels. Two varieties were used, the *Ultrafein* and *Membrane* types. The former retained albumen and Congo red solutions. The latter were graded for porosity by the time, in seconds, required for 100 cc. of distilled water to pass through 100 sq. cm. surface, under one atmosphere excess pressure at room temperature.

The membranes were stored in the ice chest under distilled water containing a few drops of formalin. When used, a membrane was transferred to a Petri dish containing merthiolate diluted 1:1000 and, after 1 hour, washed three times in Petri dishes of sterile saline. The membrane was then transferred to the ultrafilter which had been sterilized by autoclaving. 50 cc. of saline, and then 10 cc. of plain broth at pH 7.5, were first filtered in order to wash the membrane and saturate it with adsorbed protein (3). With the coarse filters, a water pump was sufficient, but with the finer membranes, positive pressures of nitrogen up to about 50 to 75 pounds per square inch were required for efficient filtration. For convenience, the filtrate was collected in a test tube placed in the flask.

About 5 cc. of culture filtrate were then added and filtered to complete dryness. The solution being filtered was slightly alkaline, about pH 7.5–8.0, since protein adsorption errors are minimized under these conditions (4). The membrane was removed with a forceps, immersed in a tube containing 7 cc. of saline, and stored in the refrigerator. The solution, obtained in this manner from the dry membrane, was referred to as the eluate.

In a preliminary series of experiments, active culture filtrates from *B. coli*, meningococcus, and *B. typhosus* were filtered through both the coarse Membrane (average porosity of about 0.75μ) and the Ultrafein varieties. Groups of rabbits were prepared by intradermal injections of the unfiltered material and 24 hours later injected intravenously with the ultrafiltered preparations. The dose employed for the latter was 25 reacting units, calculated from the original titers,

[1] These membranes were obtained from Pfaltz and Bauer, New York.

10 generations. Microscopic study of the membranal lesions showed the infection to be limited chiefly to the ectodermal epithelium and to a slight extent to the entodermal epithelium. No tendency for infection of the mesodermal elements of the membrane was observed.

Lesions from the 10th generation of the virus propagated in the chorio-allantoic membrane were carefully triturated in a sterile mortar with 10 parts of normal saline. This suspension was centrifuged at low speed for several minutes to throw down the coarser particles. The supernatant material was tested in infusion broth and in anaerobic deep meat infusion for bacterial contamination, and was found to be sterile.

2 or 3 day old chicks were used for intracerebral inoculation. The bacterially sterile virus suspension was injected intracranially in 0.05 cc. amounts by means of a size 24 needle fitted to a tuberculin syringe. Vaseline kept just above the melting point was first applied to the feather down over the bead to prevent infection of the skin at the site of injection. Immediate death from injury due to the injection often resulted. Of 10 or 12 chicks inoculated 6 or 8 would usually survive without immediate severe reactions.

Symptoms from the infection usually made their appearance on the 4th or 5th day after inoculation. By the 7th or 8th day the majority of the chicks were dead, or in the last stages of the disease.

The virus was carried through serial intracerebral passage in brain tissue by sacrificing chicks in the last stages of the disease. The brains were removed aseptically and triturated in a sterile mortar with about 5 volumes of sterile normal saline. After we were assured of the bacterial sterility of this suspension by appropriate culture in aerobic and anaerobic media another lot of baby chicks was inoculated in the manner described. The virus was thus propagated in pure culture through 14 intracerebral passages at which point the series was terminated.

Material for microscopic study was collected from chicks in the last stages of the disease, *i.e.* at the 7th and 8th day following inoculation, as well as on the 3rd, 4th, 5th and 6th day of the infection. Normal chicks of the same age were used for comparison and control. The entire heads of the chicks were removed and fixed *in toto* in Zenker's fluid containing 10 per cent acetic acid. Good fixation and complete decalcification of the skull bones was usually effected within 48 hours. Blocks for paraffin embedding were cut through the entire head in cross section. Sagittal sections were also made from several samples. By this procedure the meninges remained intact and practically all the regions of the brain as well as the surrounding structures of the cranium could be examined microscopically. Sections were usually stained with hematoxylin and eosin, dehydrated in alcohol, cleared in xylol and mounted in balsam. A few samples were stained by Ranson's pyridine silver method and others by osmic acid for fat.

In Table I the plan of the entire experiment is graphically reproduced, showing the number of chicks inoculated, the course of the

was also positive. Membranes of lower porosity were negative at all of the dilutions tested. The filtration end-point, therefore, appeared between membranes of 100 to 120 seconds, which represented an average porosity of about 50 to 100 $m\mu$.

For comparative purposes a bacteriophage for *Bacillus dysenteriae* (Flexner) was filtered through membranes of 110 and 130 seconds. In both cases, the bacteriophage was recovered practically unchanged in the filtrates. It appears, therefore, that the active principles of the phenomenon under investigation have a larger particle size than the bacteriophage employed in these experiments.

TABLE II

Ultrafiltration of Meningococcus 44B. Culture Filtrates through Zsigmondy Membrane Filters of Graded Porosities

Preparation No.	Porosity	Reactions in rabbits injected at 1:10 dilution
	sec.	
Ch. 263	60	3/0
Ch. 280	65	3/0
Ch. 317	100	2/1
Ch. 319*	110	0/3
Ch. 239	120	0/3
Ch. 313†	130	0/3

* Injected at dilution 1:5, this preparation was positive in 2 out of 3 rabbits.
† Injected at dilution 1:5, this preparation was negative in 3 rabbits.

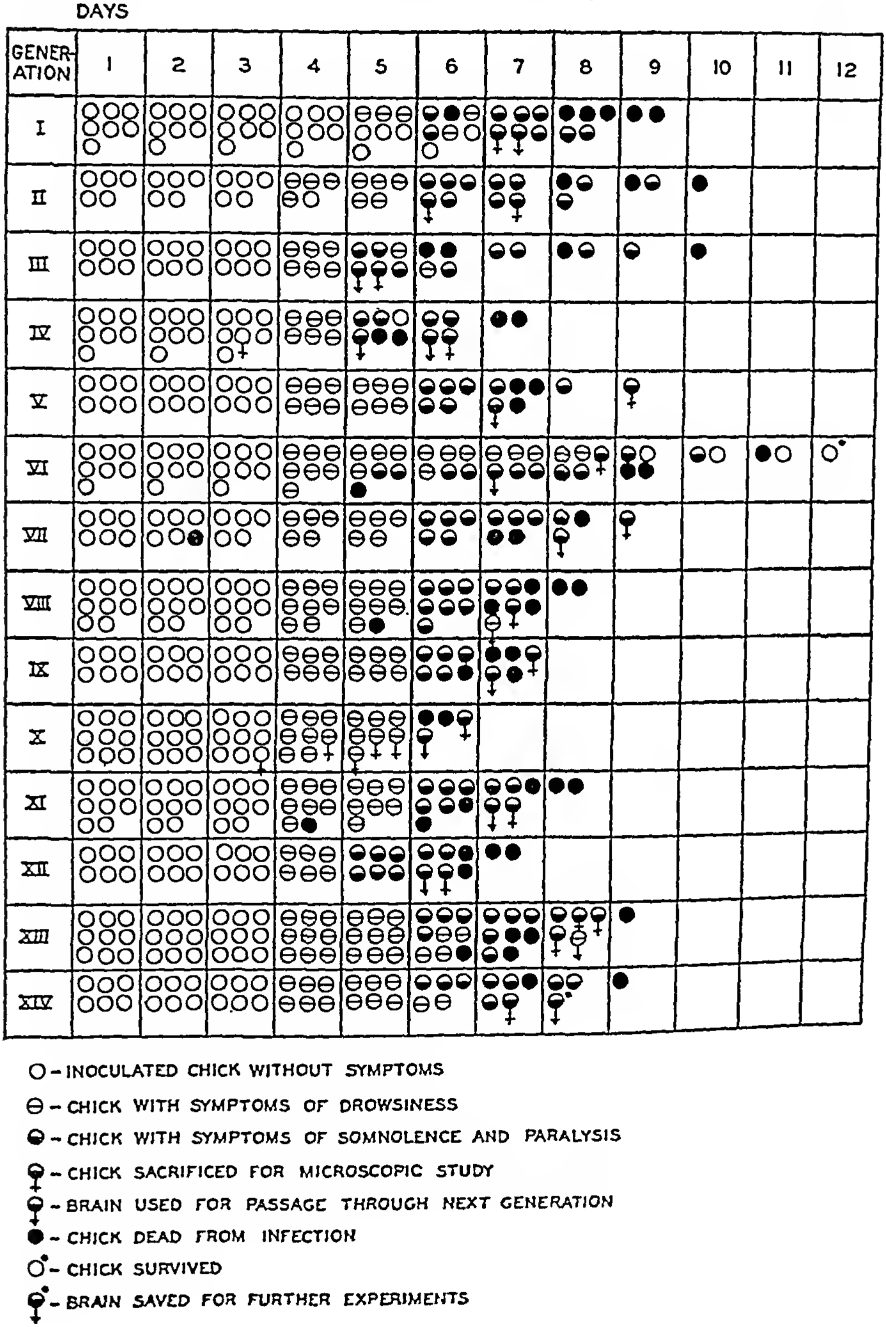
2. Fractional Precipitation with Ammonium Sulfate.—In previous work (5) "agar washings" filtrates of *B. typhosus* were concentrated and preserved as dry powders by completely saturating them with ammonium sulfate, dialyzing through parchment, and evaporating *in vacuo*. In the present investigation, fractional precipitation of other culture filtrates was conducted.

After preliminary trials, using meningococcus "agar washings" filtrates, and *B. coli* filtrates of synthetic medium broth cultures,[2] the most practical procedure appeared to be separation at one-third, two-thirds, and complete ammonium sulfate saturation, the fraction obtained at two-thirds saturation being particularly active. The

[2] These filtrates were prepared by Eli Lilly and Company, Indianapolis.

TABLE I

The Experimental Work with Intracerebral Inoculation of Chicks with Fowl Pox Virus



of the horse for a period of 5 months with group III "agar washings" filtrates.) The same serum failed to neutralize this number of reacting units of the original filtrate.

TABLE III

Fractionation of Meningococcus 44B. Culture Filtrates with Ammonium Sulfate at One-Third, Two-Thirds, and Complete Saturation

Volume of original culture filtrate	Total reacting units	Preparation No.	$(NH_4)_2SO_4$ saturation	Volume after dialysis	Dilutions injected	Reactions in rabbits	Mean titer (units per cc.)	Total reacting units	Yield	Precipitation with N. Y. Board of Health horse serum	Neutralization of 40 reacting units with monovalent group III horse serum
cc.				*cc.*					*per cent*		
		F.140			1:1500 1:1700	3/0 0/3	1600			1:16	N.N.*
25	40,000	Ch.178	1/3	15	1:160 1:800	2/1 0/3	500	7,500	19	1:8	
		Ch.179	2/3	16	1:800 1:1000 1:2000	3/0 1/2 0/3	1500	24,000	60	1:64	C.N.†
		Ch.180	Complete	20	1:124 1:400	1/2 0/3	250	5,000	13		
50	80,000	Ch.199	1/3	38	1:100 1:419	1/2 0/3	250	9,500	12		
		Ch.201	2/3	45	1:1100 1:1500	2/1 0/3	1300	58,500	73		C.N.
		Ch.203	Complete	48	1:425 1:100	0/3 0/3	<100	<4,800	<6		

* N.N. = no neutralization.
† C.N. = complete neutralization.

3. Studies on Isoelectric Properties.—

(a) Capillary Analysis.—Attempts to determine the isoelectric point of the active principles were first made by the method of capillary analysis. A simple technique for detecting the electric charge

disease and the material collected for microscopic study. It will be seen that 96 chicks survived the immediate effects of the injection. Of these, 22 were sacrificed at various stages of the disease for microscopic study and 14 were used as material to carry the virus through successive passages. Of the remainder all but one died from the resulting infection.

Clinical Course of the Disease

During the first 3 days following intracerebral injection of the virus the chicks show no symptoms. The first symptom, drowsiness, appears on the 4th or 5th day. This drowsiness increases by the 6th day to somnolence in which all interest in food and water disappears. At this time spastic paralysis of the leg muscles develops which usually reaches such an extent as to make standing impossible. On the 7th or 8th day the majority of the chicks die. Shortly before death generalized convulsions and marked opisthotonus are usually observed (Fig. 1).

Pathological Changes

Gross Findings.—Very few gross changes can be observed in the brains removed during the last stages of the infection. The dura is markedly thickened, contains small areas of hemorrhage, focal areas of necrosis and is generally quite adherent to the skull bones. The brain appears somewhat edematous and occasional scattered pin-point hemorrhages are present.

Microscopic Findings.—As will be seen from Table I, most of the material for microscopic study was collected during the last stages of the infection, although one complete series collected at daily intervals beginning the 3rd day following inoculation was obtained from which the development of the lesions could be ascertained.

The intracranial structures involved are chiefly the choroid plexus, the meninges and the perivascular connective tissues of the brain. Outside the cranial cavity, lesions are also found within the marrow cavity of the skull bones, the paranasal sinuses, mastoid cells and some of the structures of the orbital cavity.

A few general observations must be made before discussing in detail the lesions in the various structures. In the usual infection of the

The U tube contained a central hollow one-way stopcock, of about 4 cc. capacity, which could be turned so as to connect either with a narrow central chamber or with the two side compartments. The latter acted as anode and cathode chambers. The small inverted U tubes (4 mm. internal diameter x 15 cm. length) were filled, for each experiment, with 3 per cent sodium chloride containing 2 per cent agar. These tubes connected the anodal and cathodal side chambers with two non-polarizable copper electrodes. The cathode dipped into a small beaker containing 50 cc. of saturated $CuSO_4$ and a few crystals of the solid salt, while the anode was immersed into a beaker containing the $CuSO_4$ solution diluted 1:10. The electric circuit (120 volts D.C.) was similar to that described by Natarajan and Hyde (11), but with the voltmeter and lamps omitted. A small radio rheostat and milliameter were connected in series with the apparatus.

Aliquots of meningococcus 44B. filtrate F.140/T.2252, stored in a dried form in vials (6), were dissolved in various Sörensen buffers, the volume of which corresponded to that of the original culture filtrate samples. The pH values of the final solutions were determined colorimetrically. The sterilized U tube was filled with buffer solution at this pH, while the stopcock was in a horizontal position. The tube was supported on a ring-stand by clamping to the central chamber. Small loose cotton plugs were set into the side arms, by means of a copper wire, in order to prevent diffusion. The agar bridges, kept in place by corks, were immersed just below the buffer surfaces. The stopcock was turned vertically, emptied with a capillary pipette, and the buffered culture filtrate added. The stopcock was then placed horizontally and the switch turned on. In all of the experiments a current of 6.5 ± 0.5 milliamperes was maintained at room temperature for exactly 3 hours. The use of a thermostat was found unnecessary. At the end of this period, the solutions were removed and their pH values redetermined. In all cases, the original acidities were maintained.

The results of a series of experiments conducted between pH 8.0 and 2.0 are summarized in Table IV. Groups of three rabbits were prepared by single intradermal injections of 0.25 cc. of the original meningococcus filtrate and 24 hours later they received the various preparations intravenously. The solutions from the anodal and cathodal chambers were injected undiluted in a dose of 1 cc. per kilo of body weight, whereas those from the central chamber were tested for reacting potency at 1:64, which represented 25 reacting units, assuming no loss during cataphoresis.

It can be seen from Table IV that the active principles were negatively charged at pH 4.0 and above, since they migrated readily to the anode; the latter chamber became colored and turbid, while the cathode chamber remained perfectly clear. At pH 3.0 and below, however, the solutions in the anode chamber remained water-clear

skin and mucous membranes the virus induces a marked hyperplasia and swelling of the epithelial cells. The specific intracytoplasmic inclusions are distinct and easily recognized. There is no great disturbance in the spatial relationship of the infected cells to each other. Not until the lesion is well advanced, about the 9th or 10th day following inoculation, is there much necrosis of the infected cells.

In the intracranial infection there is also a marked proliferation of the cells of the susceptible tissues but this is accompanied by a rapidly developing necrosis and a profound disturbance of the shape of the infected cells and their relationship to each other. The inclusion bodies do not stand out as distinctly as in the usual lesion of the epithelium but much more intimately involve the entire cytoplasm of the cell. They seem to be much more loosely constructed and are composed apparently of numerous small refractive globules. Much of the virus seems to be forming more rapidly in proportion to the lipoid capsular material. Fat stains such as Sudan III or osmic acid in appropriately prepared material from the lesions seem to substantiate this fact.

A uniform change in the shape of infected cells takes place. They all become globular with the nucleus pushed to one side. With this change in shape separation from the surrounding cells or basement membrane occurs and the infected cells rapidly disintegrate. All types of infected cells assume this globular appearance and become separated from each other.

These processes seem to take place with relative rapidity and are present in the earliest lesions studied. The difference in the early and more advanced stages of the disease is one of extent of this rapid process rather than a slow progressive change in the infected tissues over a large area.

The choroid plexus is perhaps most profoundly affected of all the intracranial structures (Fig. 10). The normally single layer of epithelial cells has undergone rapid proliferation. Practically every cell contains inclusion bodies. Separation from the basement membrane, rapid degeneration into globular cells lying free within the ventricle and rapid disintegration take place (Figs. 11 and 12).

Marked thickening of the leptomeninges and the dura due to hyperplasia of the connective tissue cells takes place (Fig. 2). There is

Attempts to apply the method of Eaton (13), who obtained purified preparations of diphtheria and tetanus toxin by precipitation with alum and cadmium chloride, were not successful. Although adsorption with aluminum ammonium sulfate at pH 6.7 occurred, difficulties with elution were encountered.

In a former communication (2), it was reported that fractionation of culture filtrates with alcohol led to losses in activity. With preparations which were dried by freezing and evaporating *in vacuo* (6), however, it has been found that extraction in a Soxhlet apparatus could safely be conducted. In this manner, an appreciable quantity of fatty materials has been removed with ether, acetone, carbon tetrachloride, and absolute alcohol.

The stability of the active principles from meningococcus 44B. filtrate F.140/T.2252, when subjected to different hydrogen ion concentrations, has been investigated, as described below.

Samples were dissolved in buffer solutions varying from pH 3.0 to 10.0 and stored in the refrigerator for 3 months. The solutions were then tested for both skin-preparatory and reacting potency, which in all cases were retained. No significant quantitative differences were detected.

A sample was dissolved in a buffer at pH 10.0 and divided into four parts which were treated as follows: (*a*) heated for 1 hour at 56°C.; (*b*) immersed in boiling water for 15 minutes; (*c*) stored for 5 days in the refrigerator; and (*d*) in the incubator. Each solution was then tested for reacting potency at a dilution of 1:530, which represented 3 units. In all cases, two of the three rabbits injected were positive.

Two samples were dialyzed in cellophane bags at 4°C., for 1 week, against 600 cc. of buffer solutions at pH 2.8 and 10.0, respectively, the outside solutions being changed daily. The contents were removed, neutralized, and tested for reacting potency at a dilution of 1:160, which represented 10 units. All of the rabbits reacted strongly.

DISCUSSION

The data presented in this paper comprise part of an investigation on the purification of the active substances of the phenomenon of local skin reactivity to bacterial filtrates. At an early stage of this work, it became clear that further knowledge of their physicochemical properties would be of aid in understanding their general nature, as well as in facilitating their chemical isolation.

The properties of the active principles thus far investigated in these laboratories point to their protein-like character, formerly suggested

marked focal necrosis in many areas surrounded by a zone of cells of which the majority are globular in shape and contain inclusion bodies (Fig. 4). The focal necrosis is evidently due to the rapid destruction of the cells by the virus.

Within the brain proper the capillaries stand out very prominently (Fig. 5). Hyperplasia of the connective tissue cells, of which the majority are swollen and globular in shape and contain inclusion bodies, is observed (Fig. 6). Small hemorrhages around these capillaries are frequent. This is possibly due to destruction of the vessel walls by the virus. Degeneration and necrosis of focal areas of brain substance in the neighborhood of these vessels is common and is secondary to the vascular lesions.

Focal collections of large round cells containing the specific cytoplasmic inclusions are found also within the marrow cavity of the bones of the skull (Figs. 7 and 8). The centers of these areas usually show necrosis. The exact nature of the cells involved is somewhat obscure but since they are most frequently found where active bone formation is taking place they are quite likely osteoblasts.

There is also a marked involvement of the epithelial lining of the paranasal sinuses and mastoid cells during the latter stages of the disease. Marked proliferation, with practically every cell containing inclusion bodies, and a rapidly developing necrosis, characterize the infection of these structures.

Within the orbital cavity the connective tissues of the sheaths of the extraocular muscles show the same general changes as found in the meninges. The infection can be traced back in successive sections along the sheath of the optic nerve along which route it has apparently spread from the initial foci in the brain (Fig. 3).

Careful study of the neurons and supporting glial cells of the brain proper presents no evidence of actual infection of these cells by the virus, therefore there is no indication of an acquired neurotropism. The ependymal cells lining the ventricles also show no changes. Such areas of necrosis or degeneration which are present in the brain are secondary to the infection of the meninges and the perivascular lesions or are directly due to injury produced by the inoculating needle.

There is a minimum of inflammatory reaction observed in all the lesions described. Where a considerable amount of necrosis has

Ultrafiltration through Zsigmondy filters of graded porosities has shown that the active substances are retained by membranes finer than 100 to 120 seconds, whereas coarser ones readily permit their passage. The average porosity of this filtration end-point represents a particle size of about 50 to 100 mμ.

When fractionally precipitated with ammonium sulfate, most of the activity of a culture filtrate was concentrated in the two-thirds saturated portion.

Isoelectric properties were studied by means of capillary analysis and cataphoresis. At pH 3.0 and below, the substances suspended in the culture filtrates migrated to the cathode; activity in this chamber, however, could not be demonstrated. At pH 4.0 and above, reversal to the anode occurred, as the active materials became negatively charged and readily migrated to this chamber. The isoelectric point, therefore, was considered to be between pH 3.0 and 4.0.

Preliminary experiments on adsorption, extraction, and pH stability have been described.

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occurred a moderate number of actively phagocytic mononuclears are present. Polymorphonuclear leukocytes are present in very small numbers.

Microscopic study of the visceral organs of several of the infected chicks reveals no lesions. The process is evidently quite local in its development.

DISCUSSION

The pathological lesions produced in chicks by the intracerebral injection of the virus of fowl pox are of interest from two points of view. In the first place the injection produces an experimental disease with the virus which differs markedly from the spontaneous or the usual experimental infection of the skin and mucous membranes. The lesions of this disease are essentially an infection of the meninges, the choroid plexus, the paranasal sinuses, the mastoid cells and of the marrow of the cranial bones. It is almost invariably fatal and presents a uniform development of the symptoms of somnolence, spastic paralyses, convulsions and death.

The symptoms of the disease can be definitely related to the development of the infection of the meninges, perivascular tissues of the brain and the choroid plexus. The widespread meningeal involvement accounts for the meningismus observed. The somnolence or stupor are perhaps expressions of the spread of the infection and alterations in intracranial pressure. The secondary involvement of the brain proper may account for the progressive development of the spastic paralyses.

In the second place this method of propagation has produced marked changes in the behavior of the virus. What the actual nature of the conditions in this environment is which is responsible for this change is not readily amenable to analysis. However its effect on the virus can be observed in the types of cells involved and the nature of the changes produced in these cells.

A marked increase in the virulence of the virus for epithelial cells is one expression of this change in behavior of the virus. This is most clearly seen in the involvement of the choroid plexus. Not only a rapid proliferation, but a rapid destruction of these susceptible cells

EXPERIMENTAL

1. Production of Active Principles of Bacillus coli, Meningococcus 44B., and Bacillus typhosus Grown in Broth Media Diffused through Cellophane.—The technique described by McClean (1), who grew staphylococcus in a diffused fluid medium, was first used, but contaminations of the broth outside the cellophane membrane were

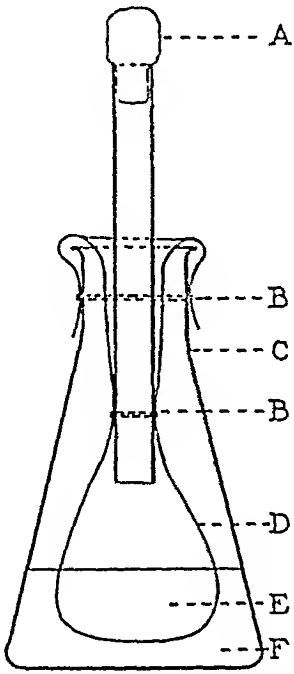

CHART 1. Apparatus for cultivation of bacteria in diffused broth medium. (A) Cotton plug. (B) Rubber bands. (C) Flask. (D) Cellophane bag. (E) Saline. (F) Broth.

frequently encountered. A somewhat modified procedure was, therefore, developed as described below (see Chart 1).

A sheet of plain transparent "Cellophane,"[1] No. 600, (50 x 50 cm.) was cut circularly and thoroughly wetted in a large evaporating dish. The membrane was drawn evenly around a glass tubing (150 x 18 mm.) to form a bag, and

[1] We wish to express our thanks to E. I. Du Pont De Nemours and Company, Cellophane Division, New York, for their cooperation in supplying us with various grades of cellophane.

is effected by the virus. This increase in virulence is perhaps due to an acquired capacity for rapid formation of the virus within these cells.

On the other hand the virus has acquired the capacity for infecting cells of mesodermal origin. The connective tissue cells of the meninges and the perivascular tissues as well as mesenchymal elements of the bone marrow are widely affected by the virus. In the natural or experimental disease in which the epithelium of the skin and mucous membranes of the upper respiratory tract are the site of the disease this feature does not appear to be prominent. Reischauer (11) in a detailed study of the natural disease has observed an occasional inclusion body in the connective tissue and cartilage cells of the orbit.

The capacity for infecting nervous tissue *per se* in the sense of a true neurotropism does not develop. Our observations give no indications which might explain why this tissue is refractory to infection by the virus.

The fact that young chicks instead of mature hens were used in this study may account for the type of lesions obtained. Those produced by Levaditi and Nicolau (8) in mature hens apparently were of a much milder nature. This problem however deserves further experimental investigation.

In the third place the virus has acquired in this environment the property of similarly altering all types of infected cells regardless of their origin so that they become spherical in shape and detached from each other. The infected cells also apparently undergo necrosis much more rapidly than the epithelial cells infected with the original dermal virus.

The variation in behavior of the virus brought about by the intracerebral environment takes place with apparent rapidity and does not seem to be greatly enhanced by repeated passage. The symptoms and lesions produced in the chicks inoculated intracerebrally at the first passage with the original strain of virus did not differ markedly from those observed at the 14th passage. In this respect fowl pox virus differs from vaccinia and yellow fever virus in that in the latter numerous passages are required to produce a maximum or fixed variation. In the case of fowl pox this maximum variation or fixation of

TABLE I

Bacillus coli, Meningococcus, and Bacillus typhosus Active Principles Produced in Diffusate Media

Inoculum	Plain broth outside cellophane bag		Samples removed from inside cellophane bag			Preparation No.	Reaction in rabbits	
	Volume	Supplemented with	Time	Volume	pH		Dilution tested	Results*
	cc.		*days*	*cc.*				
B. coli (Lewis)	1000	Glucose, glycerol, phosphate	1	150	5.8	T.2444	1:25	±/2
			2	10	8.0	T.2465	1:25	0/3
			6	50	7.0	T.2451	1:25 1:100 1:200	3/0 1/2 0/3
			11	150	7.0	T.2467	1:25	2/1
	1000	Same as above, but medium removed from bag before inoculation	2	185	7.0	T.2487	1:25	0/3
			6	185	6.0	T.2499	1:25	1/2
	100	Glucose, glycerol, phosphate	3	8	4.8	T.2416	1:25	2/1
			7	20	5.0	T.2430	1:25	3/0
	1000	" "	6	300	7.0	T.2496	1:25 1:75 1:100	3/0 1/2 0/3
	1000	—	6	380	7.5	T.2573	1:25	1/2
	1000	Glucose, glycerol	6	100	5.2	T.2575	1:25	2+/2
Meningococcus (44B.)	100	5 cc. rabbit blood; 5 cc. cocoanut milk	2	8	7.5	T.2534	1:100	0/3
			6	16	7.5	T.2538	1:100	3/0
	100	Glucose, glycerol, phosphate; 5 cc. rabbit blood†	2	10	7.0	T.2431	1:300	1/2
			6	18	7.3	T.2436	1:750 1:2000	3/0 2‡prim/1
	100	" "	2	8	7.5	T.2535	1:100	1/2
			6	22	7.0	Ch.402§	1:100 1:1000	3/0 2‡prim/1

* The numerator indicates the number of positive rabbits. The denominator indicates the number of negative rabbits. The sum total of both indicates the total number of rabbits used in each group.

† Inoculum changed to 3 cc. of a 24 hour glucose (0.7 per cent) broth culture containing 1 per cent rabbit blood.

‡ Primary reactions from intradermal injections.

§ Culture filtrate was dialyzed before testing.

new characteristics is apparently acquired on the first intracerebral passage and does not seem to increase greatly by repeated passage.

The results of these studies have served to interest us in a more detailed analysis of the variation produced in fowl pox virus by intracerebral passage. The findings of these further studies are the subject of an accompanying report.

SUMMARY

1. Intracerebral inoculation of fowl pox virus in young chicks produces a disease characterized by the development of drowsiness and somnolence 4 to 5 days after inoculation. This is followed by spastic paralysis and convulsions on the 6th and 7th day. The majority of inoculated chicks die on the 7th or 8th day.

2. The pathological lesions are found chiefly in the meninges, perivascular structures, the choroid plexus, paranasal sinuses, mastoid cells, the bone marrow of the cranial bones, and the orbital tissues. No affinity for nervous tissue *per se* develops.

3. In this environment the virus has a high virulence for the choroidal plexus epithelium and acquires the capacity for infecting cells of mesodermal origin. All infected cells of whatever origin undergo a similar structural change. Fowl pox inclusions can be demonstrated within them and they become spherical in shape and detached from each other.

4. The virus has been carried through 14 successive intracerebral passages. The symptoms and lesions in the chicks inoculated with the 14th passage showed no marked difference from those of the first passage. No enhancement of the changes brought about in the virus by the intracerebral environment seems to take place upon repeated passage.

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sate filtrates prepared in either the small or large assemblies. With meningococcus, however, the highest titers were obtained with the smaller set up (100 cc. plain broth). In all cases, a 6 day incubation period gave the best results, when, as noted above, cultures were still viable and little evidence of autolysis was seen on spreads. Inactive preparations frequently resulted after shorter periods of incubation. Although active *B. coli* preparations were obtained with the basic medium alone (T.2573), addition of glucose, glycerol, and phosphate seemed to enhance their potency (T. 2451). With *B. typhosus*, rabbit serum (T.2435), rabbit blood (T.2577), or gelatin (T.2521) were apparently required, whereas with meningococcus, the presence of blood (compare T.2586 where blood was absent) was a necessary factor.

The presence of antigenic substances in the culture filtrates was established by precipitation and neutralization titrations with specific antisera. For example, *B. coli* filtrate T.2496 (Table I) showed a precipitation titer of 1:32 with an anti-*coli* horse serum (H.492 prepared and concentrated by Eli Lilly and Company). 0.50 cc. of the serum completely neutralized 250 reacting units of the culture filtrate. It is of interest to note that the same precipitation and neutralization titer was obtained on this serum with a *B. coli* culture filtrate grown in synthetic medium broth (T.2158, formerly described (3)).

2. *Dialysis of Culture Filtrates Grown in Diffusate Media.*—The technique for dialyzing culture filtrates under strictly sterile conditions has already been described (3). In our former experiments, distilled water was used outside the cellophane bags. In the present investigation, however, 0.85 per cent sodium chloride has been employed, since the volume changes occurring in the bags were negligible under these conditions. The solutions were rendered isotonic during the dialysis and thus ready for injection. Furthermore, the rubber stoppers and glass supports (3)[2] were replaced by rubber bands, which proved more convenient and equally efficient with respect to sterility. The total solids and nitrogen analyses were conducted as formerly described. Ash was determined by heating to a dull red in a muffle oven; constant weights were obtained in a few hours. Correction of the total solids to an ash-free basis was necessary, since saline was used in conducting the dialyses. The ash content of the dialyzed solutions was always found equal to the sodium chloride concentration used for dialysis.

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TABLE II

Total Solids and Nitrogen Analysis of Bacillus coli Culture Filtrates before and after Dialysis

Section	Filtrate No.	Description	Incubation	Total solids* per cc.		Total nitrogen per cc.	
				Before dialysis	After dialysis	Before dialysis	After dialysis
			days	*mg.*	*mg.*	*mg.*	*mg.*
A†	Ch.337/371	Diffusate medium removed before inoculation	—	15.9	0.8	1.89	0.01
Comparison of diffusate medium to broth outside cellophane bag, before and after inoculation	T.2451/Ch.336	Diffusate medium retained in bag and inoculated	6	12.1	1.3	1.98	0.17
	T.2499/Ch.373	Diffusate medium removed from bag and inoculated in Erlenmeyer flask	6	12.3	1.1	1.89	0.10
	Ch.338/372	Broth from outside bag of Ch.337/371	—	26.1	8.0	3.47	1.20
	Ch.336/367	Broth from outside bag of T2451/Ch366	6	14.3	4.0	2.23	0.59
B†	T.2486/Ch.380	Diffusate medium	2	14.6	1.4	1.98	0.18
Comparison of preparations in diffusate medium, plain broth, and uninoculated diffusate controls	T.2496/Ch.381	" "	6	14.1	1.9	2.25	0.29
	T.2515/Ch.384	Plain broth	2	15.5	6.3	2.48	0.91
	T.2532/Ch.386	" "	6	14.8	4.5	2.51	0.71
	Ch.360/374	Uninoculated diffusate (control)	2	14.8	1.6	1.90	0.18
	Ch.361/375	" "	6	16.8	1.9	2.33	0.21

* Ash-free basis.

† Different batches of broth were used for the preparations described in sections A and B, respectively.

that after *B. coli* grew inside the bag for 6 days (Ch.336/367), showed that a considerable quantity of diffusible materials was continually withdrawn during the incubation period. An approximately twofold

EXPLANATION OF PLATES

All sections were stained with hematoxylin and eosin.

Plate 40

Fig. 1. Photograph of chick 7 days after intracerebral inoculation with fowl pox virus. Marked opisthotonos and spastic paralysis of legs and wings.

Fig. 2. Meningeal lesion, 7 days. Marked cellular hyperplasia and focal necrosis. ×75.

Fig. 3. Extension of infection along the sheath of the optic nerve. 7 day lesion. ×75.

Fig. 4. High power of meningeal lesion (Fig. 2). Separation of connective tissue cells and increase in their size due to inclusion bodies. ×1000.

Fig. 5. Cerebral lesion, 7 days. Marked perivascular hyperplasia and infiltration. ×75.

Fig. 6. High power of perivascular lesion in Fig. 5. Swelling and spherical shape of cells due to infection with the virus. ×1000.

likely that if growth occurred in the diffusate, then redialysis of the filtrates of these cultures would yield preparations containing only polymeric non-diffusible substances elaborated by the growing cells, including particularly the active principles. The experiments described above have shown that this has essentially been accomplished. In this connection it is of interest to note that Pappenheimer, Mueller, and Cohen (4) have recently cultivated *B. diphtheriae* in a chemically defined synthetic medium and obtained potent toxin preparations.

The growth which occurred in the diffusate was extremely abundant and the production of the active principles very consistent. The presence of antigenic substances was established by repeated precipitation and neutralization titrations with specific antisera. Thus far, the titers of the filtrates tested were maintained for about 6 months. Tests for reacting potency were also made with various broth samples obtained from the outside of the cellophane bags, within which active preparations of *B. coli*, meningococcus, and *B. typhosus* had been produced. In all cases, the outside broth samples were completely inactive. It appears, therefore, that the active principles remained non-diffusible during their production by the growing microorganisms.

The advantages derived from the method described (*i.e.*, cultivation in diffusate media, and redialysis of the filtered cultures) are considerable. From the practical standpoint, the procedure of pooling the saline washings from hundreds of agar plates is laborious compared to the relative ease of producing equally large volumes in the diffused fluid media. It must be emphasized, however, that at the present stage of development, replacement of "agar washings" is not suggested, since the latter still yield consistently higher reacting titers. From the standpoint of further chemical investigations, however, the method is invaluable, since considerably purified starting materials are now readily available in large quantity.

SUMMARY

B. coli, meningococcus, and *B. typhosus* have been cultivated in a diffused broth medium, prepared by immersing cellophane bags containing saline into nutrient broth. An abundant growth occurred inside the bags, while the outside broth remained sterile. Under these conditions, the production of the active principles of the phe-

(Buddingh: Fowl pox virus producing meningo-encephalitis)

PLATE 41

FIG. 7. Marked focal lesion in bone marrow. 7 day lesion. $\times 75$.

FIG. 8. High power of Fig. 7. Involvement of osteoblasts with fowl pox inclusions. $\times 250$.

FIG. 9. High power of Fig. 8. Osteohlasts with inclusion bodies. $\times 1000$.

FIG. 10. Choroid plexus. Marked cellular hyperplasia. $\times 75$.

FIG. 11. High power of Fig. 10. Hyperplasia, and desquamation of choroidal epithelium with involvement of the cells with inclusion bodies. $\times 250$.

FIG. 12. High power of Fig. 11. Choroidal epithelium swollen with fowl pox inclusions. $\times 1000$.

for example, the close relationship between the type-specific polysaccharides of Pneumococcus Type II, and Friedländer's bacillus type B, which parallels the serological cross relationships (4), and the similar cross relationship between Types III and VIII pneumococci (5–7). Goebel (8, 9) showed that the Type III pneumococcus capsular polysaccharide is built up of molecules of glucose and glucuronic acid in a ratio of 1:1, while the Type VIII pneumococcus polysaccharide is composed of the same substances in the ratio of 7:2.[2] He believes that the basis for the immunological crossing resides in the identity of the configuration of the aldobionic acid nucleus common to both carbohydrates. Heidelberger, Kabat, and Shrivastava (10) conclude, from quantitative estimations of the homologous and cross reacting antibodies in antisera for Types III and VIII pneumococci, that it is too soon to attempt to localize the cross reaction to a definite portion of the common configuration of these molecules.

The chemical and immunological relationship of the acetylated and the deacetylated forms of Pneumococcus Type I polysaccharide (11) was further evidence of the nature of the chemical basis of cross reactions.

Immunologically interrelated polysaccharides have also been found from widely divergent sources: the blood group A substance of man and Pneumococcus Type I polysaccharide (the acetylated form only, although the failure of the deacetylated form to cross react was not due to the acetyl radical (12)); gum arabic and Types II and III pneumococci (13); other plant gums which cross react with Pneumococcus Type II (14). These gums contain uronic acid, indicating that glucuronic acid or a related substance is the common determinant group. Less complete data indicate a serological relationship between Type I Pneumococcus and a mouse virulent strain of *Escherichia coli*, probably dependent upon capsular polysaccharides. Similar polysaccharides existing in *Bacillus proteus* X-19 and *Rickettsia prowazeki* explain the Weil-Felix reaction in the serum of patients with typhus fever (16).

The production *in vitro* of antigens containing known chemical structures has thrown considerable light on these cross reactions among natural antigens (14, 17–22) and, as a result, it has been concluded that a complex chemical substance may induce more than one kind of antibody in the animal body, depending upon determinant factors such as the presence of different chemical radicals, the position of these radicals in reference to others, and the presence of highly polar groups; but the underlying mechanism is far from being completely understood.

In still other recently recorded instances,[3] where bacterial cross relationships

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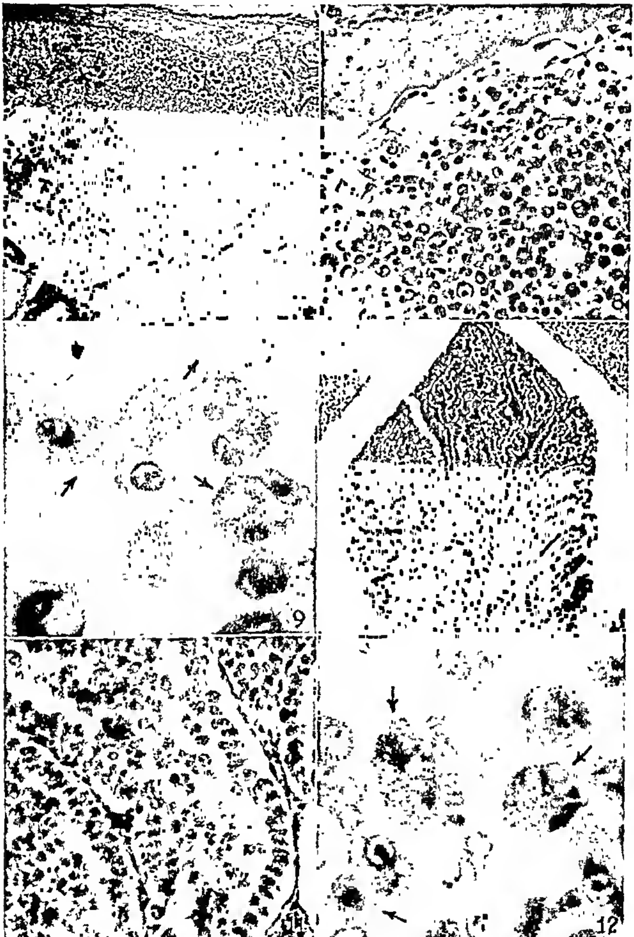
[2] Heidelberger, Kabat, and Shrivastava (10) recently gave this ratio as about 3:1.

[3] Other examples in which the serological relationships have been worked out, but in which the chemical information is less complete or lacking, are the following. (*a*) Additional subtypes of Pneumococcus Type II (23). (*b*) The relationship between yeast and Pneumococcus Type II (24). (*c*) Cross reactions of the polysaccharide of Type II Pneumococcus with antiserum for *Pasteurella cuniculicida* (25). (*d*) The relationship of *Bacterium aerogenes* to Pneu-



(Buddingh: Fowl pox virus producing meningo-encephalitis)

TABLE I

Source of Group B Strains, Types I a and I b

Strain	Animal origin	Source of culture	Isolated
		Type I a	
O 90	Human	Scarlet fever	Aronson (Wamoscher) (31), Germany
K 107	Bovine	Udder: mastitis	*Dr. F. S. Jones, Princeton, N. J.
K 158 A	Rabbit	Vaccinia	*Dr. Frieda Fraser, Toronto, Canada
Kaufman-0	Human	?	Loewenthal (32)
Human-29-0	"	Derived from strain isolated from septicemia following mastitis	" "
H 69 B 8	"	Vagina: normal maternity case	Hare and Colebrook (33), London, England
" B 13	"	" " " "	" " " " " "
H 93 B 4	"	" " " "	" " " " " "
" B 8	"	" " " "	" " " " " "
H 132 C	"	" " " "	Sloane Hospital, New York
S 117	Bovine	Udder: mastitis	*Dr. A. W. Stableforth, London, England
		Type I b	
K 127	Bovine	Certified milk	Brown, Frost, and Shaw (34), Chicago, Illinois
H 36 B	Human	Blood: new-born infant	*Dr. J. H. Brown, Baltimore, Maryland
H 132 A	"	Vagina: normal maternity case	Sloane Hospital, New York
" E	"	" " " "	" " " "
" G	"	" " " "	" " " "
F 4 A	"	Throat (slight angina) rheumatic fever	Hospital of The Rockefeller Institute, New York
H 69 B 1	"	Vagina: normal maternity case	Hare and Colebrook (33), London, England
" B 4	"	" " " "	" " " " " "
" B 12	"	" " " "	" " " " " "
H 93 B 6	"	" " " "	" " " " " "
" B 10	"	" " " "	" " " " " "
" B 12	"	" " " "	" " " " " "
" B 15	"	" " " "	" " " " " "

*Personal communication.

for Types Ia and Ib, which untreated showed reciprocal cross precipitin and protection reactions, lost all antibodies when absorbed with streptococci homologous to the respective type serum, but, on the other hand, lost only their ability to cross react when absorbed with a heterologous strain of the related type. Table III shows the results of precipitin tests with antisera treated in this way. The same relationship was made evident if the absorption was performed with

TABLE III

Precipitin Reactions

Reciprocal Absorption Experiment to Show That Types Ia and Ib Are Related but Not Identical*

†Extract from bacteria of	Type Ia antiserum		
	Serum untreated	Serum absorbed with (homologous) Type Ia bacteria‡	Serum absorbed with (heterologous) Type Ib bacteria‡
Type Ia	++++	−	++±
Type Ib	+++	−	−
†Extract from bacteria of	Type Ib antiserum		
	Serum untreated	Serum absorbed with (heterologous) Type Ia bacteria‡	Serum absorbed with (homologous) Type Ib bacteria‡
Type Ia	+++	−	−
Type Ib	+++±	++±	−

* See footnote to Table II.

† These extracts were partly purified chemically so that they were free of group-specific carbohydrate, C

‡ The same results were obtained when the absorption was carried out with the extracted and partially purified type-specific substances instead of the whole bacteria.

extracted, partially purified, type-specific polysaccharides from these two types of organisms. These results were confirmed with many different antisera.

Cross Protection between Types Ia and Ib.—This relationship was also established by protection tests in mice, since in the final analysis the best proof of immunological specificity is based upon protection conferred on animals, by passively immunizing them with the respective sera. Reciprocal protection tests with an excess of antiserum

A STUDY OF THE BEHAVIOR OF FOWL POX VIRUS MODIFIED BY INTRACEREBRAL PASSAGE

By G. JOHN BUDDINGH, M.D.

(*From the Department of Pathology, Vanderbilt University Medical School, Nashville*)

PLATES 42 AND 43

(Received for publication, February 28, 1938)

The pathological lesions which develop in young chicks following intracerebral inoculation with the virus of fowl pox are described in an accompanying report (1). It is also noted that the virus has been greatly modified in its behavior as a result of this procedure. A marked increase in virulence for epithelial cells, as well as an apparently newly acquired capacity for infecting cells of mesodermal origin, are the chief expressions of this modification.

A more detailed analysis of the behavior of the virus of fowl pox before and after intracerebral passage was suggested by several problems which arose from these observations. That a variant of the virus had been produced in this manner seemed evident. Whether the maintenance of this variation was dependent upon the intracerebral environment or would continue to manifest itself when the virus was introduced into another environment, preferably the skin of susceptible chicks or the chorio-allantoic membrane of chick embryos, presented a problem of particular interest. Furthermore, it seemed of importance to ascertain whether this modification could be considered as a permanent acquisition of new characteristics by the virus.

An analysis of this type also presented opportunities for discovering possible mechanisms by which variants of certain virus strains are brought about and might also indicate more or less definitely in what manner these variations express themselves.

A comparative histopathological study of the lesion produced by the virus of fowl pox was therefore undertaken both before and after intracerebral passage.

TABLE V*a*

Reciprocal Passive Protection Tests in Mice, Using Varying Amounts of Absorbed and Unabsorbed Antiserum to Show That Types Ia and Ib Are Related but Not Identical

Type Ia culture (homologous) strain O 90	Type Ia antiserum (against strain O 90)			
	0.5 cc. of serum diluted	Serum untreated	Serum absorbed with (homologous) *Type Ia	Serum absorbed with (heterologous) Type Ib
0.5 cc. 1:200 dil.	1:1	S	D 1 day†	S
" " " "	1:2	S	D 1 "	S
" " " "	1:10	S	D 1 "	S
" " " "	1:50	S	D 1 "	S
" " " "	1:100	S	D 1 "	S
" " " "	1:250	S	D 1 "	S
" " " "	1:500	S	D 1 "	S
" " " "	1:1,000	S		S
" " " "	1:1,500	S		S
" " " "	1:2,000	S		D 3 days
" " " "	1:2,500	S		D 6 "
" " " "	1:3,000	D 6 days		S
" " " "	1:4,000	S		D 3 days
" " " "	1:5,000	D 6 days		S
" " " "	1:6,000	S		D 7 days
" " " "	1:7,000	D 3 days		
" " " "	1:8,000	S		
" " " "	1:9,000	D 3 days		
" " " "	1:10,000	D 4 "		
	‡Virulence control			
" " " "	No serum	D 1 day		

Protection tests recorded in Tables V *a*, *b*, *c*, and *d* were performed as follows: Serial dilutions of serum in saline, contained in a volume of 0.5 cc., were injected intraperitoneally into white mice simultaneously with 0.5 cc. of a 1:200 broth dilution of a fresh 16 hour broth culture. Controls received this dilution of culture without serum. Additional controls which received from 10^{-1} cc. through 10^{-8} cc. were included to determine the minimal lethal dose of the culture. The technique of this determination and of the estimation of the number of organisms inoculated was that described under Table IV.

* Strains K 158 A and K 107 were used as typical Type Ia strains for absorpton on account of the great difficulty of sedimenting strain O 90 in the centrifuge.

† Further tests with a larger amount of serum (0.5 cc. undiluted serum) and serial dilutions of culture, usually to a dilution containing 10^{-7} or 10^{-8} cc., resulted in the death of all mice.

‡ Additional virulence controls showed that 10^{-1} through 10^{-8} cc. of this culture killed mice.

EXPERIMENTAL

The chorio-allantoic membrane of 12 day old chick embryos and the skin of baby chicks were chosen for the comparative study of the infection induced by fowl pox virus before and after intracerebral passage. Two strains of virus were studied. The first strain was one which was used in the study of the intracerebral infection of chicks as described in the accompanying report (1). The second strain, which was obtained from another source,[1] had been propagated only in the skin of chickens. After one passage in pure culture in the chorio-allantoic membrane it was inoculated intracerebrally into chicks. The virus from the first intracerebral passage was studied for its characteristics.

The intracerebral virus was inoculated onto the chorio-allantoic membrane of chick embryos with the technique described by us for the cultivation of vaccinia virus (2). For control and comparison the original strains which had not been passed intracerebrally but had been maintained in the chorio-allantoic membrane in pure culture were used. The skin of baby chicks was also infected with both strains of virus before and after intracerebral passage.

The lesions in the chorio-allantoic membrane were collected 4, 5 and 6 days after inoculation. Those in the chick skin were collected at daily intervals from the 4th to as late as the 12th day after inoculation. It was found that the 4th day lesions in both the membrane and the chick skin provided the best material for microscopic study.

The lesions were fixed in Zenker's fluid with 10 per cent acetic acid. Embedding was done in paraffin. The sections were stained with hematoxylin and eosin.

Histology

Lesion Produced by the Virus before Intracerebral Passage.—

1. In the Chorio-Allantoic Membrane.—The fowl pox lesion in the chorio-allantoic membrane has been previously described by Woodruff and Goodpasture (3). Our observations confirm their findings. Marked hyperplasia of the epithelial cells of the ectoderm in the inoculated area occurs. The cells are swollen and in practically every one the characteristic inclusion bodies are found (Figs. 3 and 5). In the 4 day old lesion no necrosis of the infected cells is observed. The mesoderm is swollen, chiefly due to edema. A slight amount of proliferation of fibroblasts takes place. A moderate number of mononuclear leukocytes have wandered into the mesoderm. No hemorrhage or reaction around the blood vessels is seen. The entodermal layer shows focal proliferation, but no inclusion bodies are found in the entodermal cells in the 4 day lesion. Inclusions appear in these cells in the lesions at 6 days. In the older lesions in which focal necrosis of the ectodermal epithelium has occurred there is more proliferation of fibroblasts in the mesoderm. This is most marked directly beneath the necrotic foci in the ectoderm. In these areas, an occasional fibroblast is swollen and contains a globular refractile mass which resembles an inclusion.

[1] Obtained from Dr. N. J. Pyle of the Lederle Laboratories.

For this experiment the same lots of antisera used in the precipitin absorption experiment recorded in Table III, were injected into mice in dilutions varying from 1:1 to 1:30,000. Simultaneously, the animals were inoculated intraperitoneally with 0.5 cc. of a 1:200 dilution of culture which had a minimal lethal dose for mice of 10^{-6} to 10^{-8} cc. Table V*a* shows the protective power of Type Ia antiserum for mice infected with Type Ia organisms. The high protective titer of the untreated serum for the homologous strain is recorded in column 3. Mice which received this serum survived infection even though the serum was diluted as much as 1 part in 2,500. With

TABLE V*c*

Reciprocal Passive Protection Tests in Mice, Using Varying Amounts of Absorbed and Unabsorbed Antiserum to Show That Types Ia and Ib Are Related but Not Identical

Type Ia culture (heterologous) strain O 90	Type Ib antiserum (against strain H 36 B)			
	0.5 cc. serum diluted	Serum untreated	Serum absorbed with (heterologous) *Type Ia	Serum absorbed with (homologous) Type Ib
0.5 cc. 1:200 dil.	1:1	S	D 1 day†	D 1 day†
" " " "	1:2	S	D 1 "	D 1 "
" " " "	1:10	S	D 1 "	D 1 "
" " " "	1:50	S	D 1 "	D 1 "
" " " "	1:100	D 1 day	D 1 "	D 1 "
" " " "	1:250	D 2 days	D 1 "	D 1 "
" " " "	1:500	D 1 day	D 1 "	D 1 "

Virulence controls recorded in Table V*a*.
*† See Table V*a* for these footnotes.

higher dilutions the deaths were irregular, but still the animals survived for 2 to 3 days longer than the control mice, all of which died within 1 day. The animals which received serum absorbed with bacteria of the homologous type all died within 1 day. In further tests with larger amounts of this absorbed serum, it was found impossible to protect mice against even one minimal lethal dose of the homologous culture. When, on the other hand, the serum was absorbed with bacteria of the heterologous type, Ib, the protective capacity of the absorbed serum against Type Ia infection was not significantly reduced.

Both dermal strains of virus studied induced essentially the same lesion when inoculated on the membrane.

2. In the Chick Skin.—The fowl pox lesion in the chicken skin has been described by numerous investigators. Both strains of virus in the present study induced lesions which could not be distinguished from each other. Hyperplasia of the epithelium of the skin and feather follicles with the presence within these cells of the typical fowl pox inclusions is characteristic (Fig. 1). In lesions older than 8 days, where necrosis of the infected epithelium is present, hyperplasia of the underlying connective tissue takes place. Within an occasional fibroblast a refractile body resembling an inclusion can be observed.

The older lesions in the chick skin show a greater amount of inflammatory reaction than is observed at any stage of the infection of the chorio-allantoic membrane. This is possibly accentuated by secondary infection which is favored by the ulceration which sets in during the later stages of the disease.

The Lesion Produced by the Virus after It Has Been Passed Intracerebrally.—

1. In the Chorio-Allantoic Membrane.—The 4 day membranal lesion following inoculation with the virus which has been passed intracerebrally differs greatly from that produced by the original strains. (Compare Figs. 3 and 4.) Instead of hyperplasia, a widespread necrosis of the ectodermal epithelium is observed. At the advancing edge of the lesion where active infection of the ectodermal epithelium is present there is a slight hyperplasia of these cells. Inclusion bodies can be found in practically every cell in these areas. The infection affects these cells profoundly, they become rounded in shape and detached from one another (Fig. 6). Necrosis seems to set in rather soon after the cells are invaded by the virus. As the inclusion increases in size, the cells separate, become globular and break up, setting free many of the bodies which can be recognized as inclusions. This type of change is observed in the infection with the virus of the epithelial cells of the choroid plexus of the chick (1).

The mesoderm is also much more profoundly affected by the virus of intracerebral derivation than by the original strains. Although much of the thickening of this layer is due to edema there is considerable proliferation of fibroblasts, and a larger number of mononuclear phagocytes are present. The majority of the fibroblasts in the mesoderm are found to be infected with the virus and contain typical inclusions. Many stages in the infection of these cells can be observed. At first the fibroblasts show their usual shape, having an oval nucleus and long branched cytoplasmic processes. The first evidence of infection is the swelling and opaque appearance of the cytoplasm of the body of the cells. In this opaque area a small refractile round body can usually be seen. This rapidly enlarges and the entire cell becomes more swollen and spherical. The nucleus is pushed to one side and becomes shrunken and pyknotic (Figs. 9 and 10). Following this, the cell rapidly disintegrates.

The blood vessels in the mesoderm also show marked changes. Their walls are greatly thickened by proliferation of fibroblasts. Many of these cells contain inclusions. The endothelial cells lining the vessels are often irregular and swollen.

mice varied on different occasions from a minimal lethal dose of 10^{-5} to 10^{-7} cc., while that of strain O 90 rarely fell below 10^{-8} cc. Tested against the heterologous strain, H 36 B, the Type Ia antiserum absorbed with either Type Ia or Type Ib bacteria failed entirely to protect mice against infection with this strain.

These absorption experiments demonstrate that the homologous Type Ia bacteria remove from Type Ia antiserum the antibodies which protect against either strain, while the heterologous Type Ib strain removes only that antibody which protects against itself. This agrees with the evidence afforded by the precipitin reaction.

Absorption of Protective Antibodies: Type Ib Antiserum.—Tables V*c* and V*d* show the results of similar experiments with Type Ib antiserum. In Table V*c* there are shown the results of passive protection tests in mice injected with absorbed and unabsorbed Type Ib antiserum and inoculated with the heterologous Type Ia strain, O 90. This serum had a relatively low titer of protective antibody for the heterologous strain, although it protected mice against infection with the homologous strain, H 36 B, even when the serum was diluted 1:10,000 (*cf.* 3rd column of Table V*d*). Absorption with the homologous Type Ib strain removed the protective antibodies for both Types Ia and Ib (column 5, Tables V*c* and V*d*), in marked contrast to the absorbing capacity of the heterologous Type Ia strain, which removed the antibody for itself only (column 4, Table V*c*) and not that for the strain homologous to the serum (column 4, Table V*d*). In this last titration the end-point for protection against the homologous strain, H 36 B, was not reached, but obviously the protective power of this lot of Type Ib serum absorbed with the heterologous Type Ia strain was still high.

DISCUSSION

The results of the cross precipitin and cross protection tests with these reciprocally absorbed antisera and the control untreated antisera prove that one of the serological types previously identified within group B streptococci really comprises two types, now designated as Types Ia and Ib.[4] These are closely related, as shown convincingly by the large amount of cross protection with unabsorbed sera for the two types; but they are not identical, even though so closely related

Margination of thrombocytes is often observed, giving evidence of injury to the vessel wall (Fig. 7). Hemorrhages from the veins are common. Close examination of the endothelial cells lining the vessels show them to be swollen, often detached and globular and containing typical inclusion bodies (Fig. 8). The infected endothelial cells undergo necrosis and a breakdown of the vessel wall takes place, resulting in the frequent hemorrhages observed.

The entodermal layer of the membrane also gives evidence of the increased invasive power of the virus. There is marked hyperplasia and widespread involvement of the cells by inclusion bodies. The infected entodermal cells also become detached from one another and spherical in shape after they are infected.

2. *In the Chick Skin.*—In this tissue also the difference in the lesion produced by the virus after intracerebral passage in contrast to that produced by the strain originally is very marked. (Compare Figs. 1 and 2.) The epithelial cells show widespread necrosis instead of the hyperplasia usually encountered in fowl pox infection of the skin. At the edges of the lesion and in the depths of the feather follicle the epithelial cells are seen to contain the typical inclusions. After invasion by the virus and with the increase in size of the inclusion body the cells become spherical in shape and detached from each other. The infected feather follicles show the difference in behavior of the intracerebral virus from the original strain particularly well. Following infection with the original strain these structures stand out sharply and the component cells retain their relationship to each other even after all of them are swollen and contain inclusion bodies. The original pattern of the feather follicle is preserved. In the follicles infected with the intracerebral virus the entire structure is destroyed and only a clump of round separated cells, each filled by an inclusion, can be recognized (Figs. 1 and 2).

The subcutaneous connective tissue shows numerous foci of necrosis. At the edges of these areas numerous connective tissue cells contain inclusion bodies. The walls of the smaller arterioles are thickened and surrounded by an exudate consisting chiefly of large mononuclears. The endothelial lining is swollen and irregular but no definite evidence of inclusions has been found in these cells. Small areas of hemorrhage are frequent.

The inflammatory reaction is quite intense and both polymorphonuclear and mononuclear phagocytes are present in large numbers. The rapid necrosis of the cells following infection by the virus favors secondary infection which is partly responsible for the intense inflammatory reaction.

Effect of Serial Transfer in the Chorio-Allantoic Membrane on the Intracerebral Strain of Virus

The virus from the 15th intracerebral transfer in chicks was subsequently propagated in the chorio-allantoic membrane through 7 generations. Membrane to membrane inoculations were performed with 4 day lesions. Lesions from each generation were prepared

cross reactions. The latter viewpoint is probably the more tenable, since in neither case has it been possible to separate the type-specific substance chemically into two fractions, one common to the two types and one strictly specific for the type in question, or into fractions of different reactivities. Further study of these relationships is being made by growing each organism in media containing respectively antisera for each of these related types. The preliminary indications are that, under certain conditions, the Type Ib strain, H 36 B, can be made to produce an antigen which is almost strictly specific for that type, but the results with the Type Ia strain are somewhat different and too incomplete for inclusion in this report.

The foregoing observations are of interest in connection with a number of experiments reported recently in which it has been found that, although cross immunological reactions between bacterial and other naturally occurring substances usually indicate genetic relationships, as is true in the present instance, it also sometimes happens that no such consistent relationship is apparent. However, where fairly complete chemical data are available, it has been found that serological similarities are always based on chemical likenesses whether the determinant substances are derived from related or from unrelated sources.

A chemical and immunological investigation of particular importance as a pattern in determining the nature of such partial cross immune reactions among bacteria, was the formation of effective antigens from α and β glucosides by linking them to serum proteins (17 *a*, *b*). Avery, Goebel, and Babers were thereby enabled to furnish an example of substances of known structure which simulated the serological behavior of such bacterial polysaccharides as those derived from Friedländer's bacillus type B, and Pneumococcus Type II. The only difference in the two synthetic antigens studied was in the position of the glucoside linkages, but this was reflected in their serological reactions. Other studies which show a chemical basis for serological relationships have already been discussed, as well as the conclusions of several investigators that a single complex chemical substance may induce the formation in animals of more than one antibody.

With these facts in mind, it is not surprising to find another example

for microscopic study to determine whether the characteristics acquired upon intracerebral passage were maintained. It was found that the virus behaved in the same manner after 7 generations in the membrane as it did after inoculation on the membrane directly from the infected chick brains. It would seem from this that once these characteristics are acquired by the virus they are permanent and are maintained when cultured away from an intracerebral environment.

Number of Intracerebral Passages Required to Produce the Change in the Behavior of the Virus

In order to ascertain whether a prolonged period of intracerebral propagation was required to establish the change in the behavior of the virus or whether it was rapidly acquired, the second of the original strains of virus was propagated through one intracerebral passage and then inoculated onto the chorio-allantoic membrane. It was found that this strain of virus had the same characteristics as that cultured through 15 intracerebral passages. This strain was then propagated through 5 serial transfers in the chorio-allantoic membrane with no observable change in its characteristics. The virus evidently acquires its new properties rapidly and permanently.

Effect of Culturing the Original Virus in the Chorio-Allantoic Membrane in the Presence of Normal Chick Brain Tissue

In order to rule out or establish the possibility that the presence of normal brain substance alone, without actual intracerebral passage, might produce the change in the behavior of the virus, equal amounts of membranal lesion containing the original virus and normal chick brain were carefully triturated and inoculated onto the chorio-allantoic membrane in the usual manner. Microscopic study of the developing lesion showed that the presence of brain substance did not influence the behavior of the virus. No tendency toward increase in virulence for ectodermal epithelium or invasion of the mesodermal elements of the membrane was observed.

DISCUSSION

Answers to some of the problems raised at the onset of these experimental observations appear evident. By means of intracerebral

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passage the virus of fowl pox can be so modified that a definite variation results. This variation continues to express itself in the behavior of the virus when it is propagated away from intracerebral conditions in the skin of chicks or in the chorio-allantoic membrane of the chick embryo. The capacity for causing a rapidly developing necrosis of infected epithelial cells of the chick skin, as well as of the ectodermal epithelium of the chorio-allantoic membrane, seems to be the expression of an increase in virulence. Furthermore the marked propensity for infecting mesodermal cells which was evident in the intracerebral lesions is maintained when the virus is propagated in these tissues removed from the intracerebral environment. The uniform change in form which all types of cells undergo following infection with the virus is another expression of the change in behavior of the virus.

That such a variation in the virus should take place upon intracerebral passage is not unprecedented. As mentioned in the accompanying report (1) such variants have been produced in much the same manner with the viruses of vaccinia and yellow fever. What is of interest however is the fact that the intracerebral variant of fowl pox virus expresses this modification in behavior in its relationship to specific types of cells comparable to that of the so called neurotropic strain of vaccinia virus. In a previous study (4), we were able to show that in the chorio-allantoic membrane a strain of neurotesticular vaccinia was distinguished from a dermal strain by its increased virulence for epithelial cells and also in its capacity for infecting cells of mesodermal origin. The capacity of the neurotesticular strain of vaccinia and the intracerebral strain of fowl pox virus to infect the endothelial cells of blood vessels is an analogous phenomenon which points perhaps to some fundamental process which is responsible for this variation in each of these viruses.

The variation produced in fowl pox virus in this manner is obviously the result of environmental factors. Findlay (5), has recently reviewed the many variations which occur in animal viruses. He discusses the ways in which changes of environment might act in bringing about a variation of a virus. Sudden hereditary variations analogous to mutations, the selective action of an environment upon an already heterogenous population or temporary but reversible fluctuations in the sense of a *Dauermodifikation* are considered by him to be the ones most likely to occur in viruses.

bones of the adolescent animal. In the rat whose epiphyses never close, it was found (2, 3) that when the temperature of the outlying bones of the tail was elevated to the deep body level, red marrow returned to the previously fatty metaphyseal regions.

In the present experiments it was found that colloidal thorium dioxide injected intravenously remained fixed in the reticulo-endothelial cells, without causing noticeable effect on the animal. The tenacity with which it is held locally and the ease of identification of this substance enabled recognition of new areas of marrow growth, so that the growth pattern stood out plainly.

Colloidal thorium dioxide injected intravenously was found to accumulate in the reticulo-endothelial system by Oka (4) and Radt (5). Following relatively large doses, the distribution of the metal in the macrophages of bone marrow makes this structure visible by x-ray examination of the living rabbit, where thorium is concentrated (13).

Materials and Methods

37 rabbits, mostly young, were injected intravenously with an electronegative thorium dioxide sol (thorotrast[1]) containing approximately 25 per cent by weight of ThO_2 in submicroscopic particulate form. The dosage administered was relatively large, 4 to 6 cc. at 2 day intervals until each rabbit had received 10 to 15 cc. per kilo; no toxic effects were observed with these amounts but injections of 8 cc. proved fatal on 2 occasions. Before and after injection the venous blood was studied repeatedly from the standpoint of erythrocyte, leucocyte, and hemoglobin content. The cells were counted by the standard pipette and counting chamber method and the oxygen capacity of the blood was determined by the Stadie-Wu method (14). X-rays were made of the extremities each week. Where there are many macrophages, the marrow is clearly visible to x-ray after 12 cc. per kilo have been injected. At intervals of 1 to 118 days the rabbits were killed; following 10 per cent formalin fixation and decalcification with 5 per cent nitric acid, x-ray films were again made. Since thorium dioxide is insoluble in this acid a shadow of the metal as it is deposited in the marrow cells was obtained. Subsequently histological sections were made with a paraffin technique. Unna-Pappenheim plasma cell stain was useful in identifying the thorium dioxide in the tissues.

In 6 rabbits, 1 to 2 months of age, under ether anesthesia, small bits of sterile chromium steel wire were inserted into the bone marrow of lower femora and upper

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[1] We are indebted to the manufacturers, the Heyden Chemical Company, for this material.

From our observations it seems evident that the variation is due to a rapidly developing change, rather than to the selective action of the new environment. It has been found that one intracerebral passage is sufficient to bring about this type of variation. As far as our experiments have gone the modification seems to be a fixed hereditary one. The evidence is as yet not sufficient to conclude whether it may be regarded in the sense of a true fixed mutation or possibly a *Dauermodifikation* from which a gradual reversion to the normal strain might be expected. This problem is still under investigation.

Variations in bird pox viruses have been observed chiefly as a result of passage in species different from those in which they originated. The various strains have shown marked differences in pathogenicity for different species of birds. A variant produced by propagation in a different tissue of the same host has to our knowledge not been observed. The easily recognizable intracellular changes produced by the fowl pox virus offer an excellent opportunity for further study of the mechanism underlying the formation of such variations and the results of such changes in both the virus and the infected host.

SUMMARY

1. Intracerebral transfer in chicks of fowl pox virus produces marked changes in its behavior when studied in the chorio-allantoic membrane of chick embryos and in the skin of baby chicks.

2. A great and persistent increase in virulence for epithelial cells, characterized by rapid necrosis instead of proliferation and hyperplasia, is acquired by the virus propagated intracerebrally.

3. An affinity for cells of mesodermal origin including endothelial cells of blood vessels, and an increase in affinity for entodermal cells is acquired by the virus propagated intracerebrally.

4. The intracerebral virus causes a uniform morphological change in all types of cells, in that the infected cells rapidly become spherical in shape, detached, and desquamated followed by necrosis.

5. One intracerebral passage is sufficient to produce this change in the virus.

in rabbits soon after the injections had been completed contained many thorium-laden cells, but where weeks or months had elapsed since the last injection, this area was entirely devoid of the metal.

Examination of the epiphyseal region 1 or 2 days after completing the thorium injections elucidates the mechanism of marrow growth. It was found that each cartilage column was faced at the aspect bordering on the marrow with a thorium-laden macrophage (Fig. 10).

From those experiments where bits of chromium wire were inserted, followed by thorium dioxide injections, a similar opinion was derived. The wires served as fixed points in the bone marrow and the epiphyseal plates grew away from these points and new thorium-free marrow was formed in the interval.

Width growth is likewise visible in similar x-ray and histologic studies of the long bones. At the periphery, the marrow was found packed with thorium-laden macrophages following recent injection; in experiments of longer duration since injection, the thorium-containing older marrow occupied a more central position in the bone and was surrounded by a rim of thorium-free marrow.

The Normal Growth Pattern of Liver and Spleen.—These organs like the bone marrow have a sinusoidal circulation and are rich in macrophages. X-ray and histological studies of liver and spleen show that growth here is interstitial and not polarized as in the marrow (Fig. 7).

DISCUSSION

It is now possible to correlate the observations made on normal marrow growth with some previous observations.

It was shown (2) that when a graft of avascular precartilage was transplanted to any region of the donor it became vascularized with endothelium having a phagocytic character. The primitive precartilage of the tail of a newborn rat transplanted to various tissues, for example the subcutaneous tissue of the abdomen, the peritoneal cavity, or the pleura, became invaded with blood vessels lined by macrophages instead of common non-phagocytic endothelium. The nature of the graft determined that any vessel entering would develop reticulo-endothelial system properties; this character is not inherent in the vessel as such but is conditioned by the graft.

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EXPLANATION OF PLATES

All sections were stained with hematoxylin and eosin.

Plate 42

FIG. 1. Lesion in chick skin following inoculation with dermal strain of fowl pox virus (4 day lesion). ×65.

FIG. 2. Lesion in chick skin following inoculation with fowl pox virus after intracerebral transfer. Marked necrosis of epithelium and intense reaction (4 day lesion). ×65.

FIG. 3. Lesion in chorio-allantoic membrane following inoculation with dermal strain of fowl pox virus. Hyperplasia of ectodermal epithelium (4 day lesion). ×65.

FIG. 4. Lesion in chorio-allantoic membrane following inoculation with fowl pox virus after intracerebral transfer. Necrosis of ectoderm, marked reaction in mesoderm and entoderm (4 day lesion). ×65.

FIG. 5. High power of Fig. 3. Hyperplasia of ectodermal epithelium and the presence of inclusion bodies. ×300.

FIG. 6. High power of lesion in ectodermal epithelium of chorio-allantoic membrane infected with intracerebrally passed virus. Rounded form and separation of epithelial cells. ×300.

Growth in width of the marrow has been shown in these experiments to be essentially a peripheral effect occurring in the region where bone is being removed to enlarge the marrow cavity. In view of the observations of Beard and Rous (12) of the tendency of liver macrophages in tissue culture to scatter and climb, it is possible that peripheral growth is due to some such effect occurring when the marrow cavity is enlarged.

SUMMARY

Intravenous injections of colloidal thorium dioxide were made in rabbits and moderate amounts caused no significant changes in the cellular elements of venous blood. Thorium dioxide was held locally with great tenacity in the cells of the reticulo-endothelial system in the bone marrow and showed little tendency to migrate despite drastic stimuli to the marrow in the form of anemia and plethora.

In recently injected rabbits, thorium-laden macrophages abutted each cartilage column in the direction of growth where the cartilage was being removed. In marrow which had formed subsequent to injection the macrophages were thorium-free, allowing recognition of the new tissue by x-ray and histological techniques as clear zones. The growth pattern of marrow could be detected in this way.

The bone marrow increases in length principally in the region of hypertrophic cartilage at the metaphyses and it is evident that the increase is facilitated by the presence of macrophages whose primary function is the resolution of the wasted hypertrophic cartilage cells. Growth in thickness of bone marrow occurs at its circumference. In contrast to the zonal growth of bone marrow, growth of the reticulo-endothelial system in liver and spleen is chiefly interstitial.

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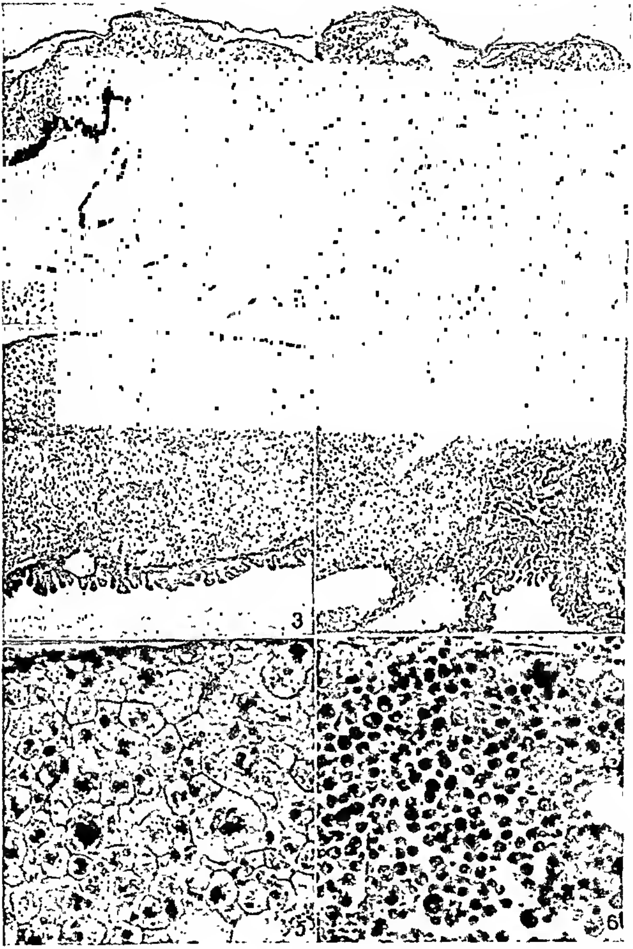


(Buddingh: Fowl pox virus modified by passage)

EXPLANATION OF PLATES

Plate 1

Figs. 1 to 5. X-rays of lower femur of growing rabbits at intervals after injection of thorotrast intravenously. The bones were decalcified in 5 per cent nitric acid before radiography. The clear zone between the epiphysis and marrow of the diaphysis represents the growth of marrow at the epiphyseal cartilage.

Fig. 1. X-ray made 1 day following injections totaling 32 cc.

Fig. 2. X-ray made 42 days following injections totaling 28 cc.

Fig. 3. There were two series of injections, the first 41 days and the second 20 days before this x-ray was made. Two thorium levels may be seen.

Fig. 4. X-ray made 97 days following injections totaling 33 cc.

Fig. 5. X-rays made 108 days following injections totaling 26 cc.

Fig. 6. Similar to Figs. 1 to 5 except that the rabbit was adult. This x-ray was made 36 days after injection of 38 cc.

Fig. 7. A lobe of the liver of the same animal as in Fig. 5. Thorium reaches to the liver edge and there is no clear zone of new growth.

Fig. 8. Small pieces of chromium steel wire (densest bars) were placed in tibia and femur of this 32 day old rabbit which was then injected with 8 cc. thorotrast intravenously. This x-ray was made on completion of injection, 9 days after operation. This and Fig. 9 are x-rays made on the living animal.

Fig. 9. Same as Fig. 8, 85 days later. The epiphyses have grown away from the bits of wire and the thorium in the diaphysis, leaving a clear zone of new marrow growth. At the periphery the arrows point to the clear zone where growth in width has taken place.

Figs. 10 to 12. These illustrations are photographs of sections stained lightly with Unna-Pappenheim plasma cell stain.

Fig. 10. The lower femoral epiphyseal cartilage 1 day following the injection of 22 cc. of thorotrast. Each hypertrophic cartilage cell bordering on the marrow cavity is faced with a macrophage containing particulate thorium dioxide. ×172.

Plate 43

Fig. 7. Vein in chorio-allantoic membrane infected with intracerebrally passed virus. Thickening of walls, irregularity of endothelial lining and margination of thrombocytes. $\times 300$.

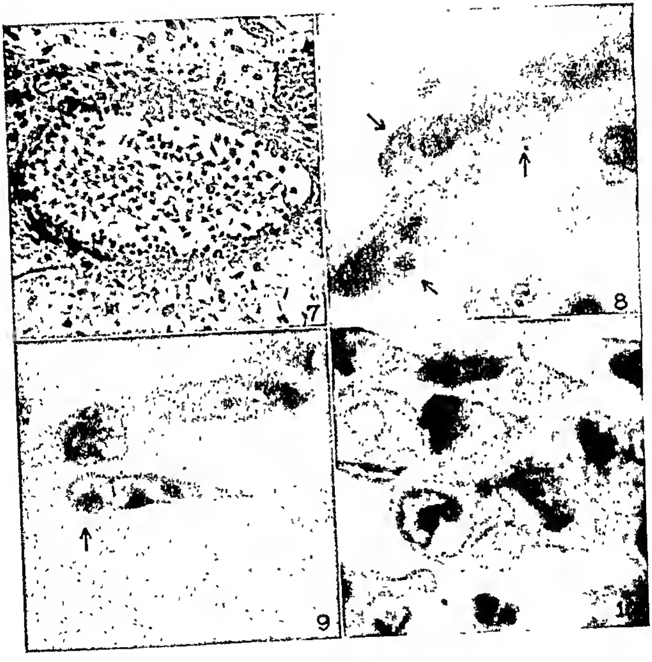
Fig. 8. Endothelial cells of blood vessel in chorio-allantoic membrane infected with intracerebrally passed virus. Extreme swelling and presence of inclusion bodies. $\times 2000$.

Figs. 9 and 10. Fibroblasts in chorio-allantoic membrane infected with intracerebrally passed virus, containing inclusion bodies. $\times 2000$.

Plate 2

Fig. 11. The lower femoral epiphysis 1 day following injection of 27 cc. of thorotrast. The dense black thorium dioxide clumps reach the epiphyseal cartilage (O). × 25.

Fig. 12. Same as Fig 11 in a litter mate 36 days following completion of injection of 26.2 cc. of thorotrast. At the left arrows point to the lower limit of the thorium, the epiphyseal cartilage (O) is at right of center and the epiphysis marrow is farther right. The thorium-free zone of new marrow growth lies between the arrows and the epiphyseal cartilage (O). × 25.



(Buddingh: Fowl pox virus modified by passage)

TABLE I

Biochemical Reactions of Fusobacteria

Microorganisms	Source	Fermentation of carbohydrates													pH in 1 per cent glucose broth		Hydrolysis of starch (2 wks.)			Hydrolysis of sodium hippurate (2 wks.)	Gelatin liquefaction	Odor	Growth in presence of bile†		Utilization of citrate	Production of indol‡	Reduction of nitrates§	Production of H_2S‖	Action on milk**	Gas from peptone water	Proteolysis							Fibrinolysin***
		Arabinose	Dextrin	Dextrose	Dulcite	Glycerol	Lactose	Levulose	Maltose	Mannite	Rhamnose	Salicin	Saccharose	Xylose	2 days	14 days	Hydrolysis*	Final pH	Benedict test for glucose				10 per cent	40 per cent							Defibrinated blood††	Coagulated blood‡‡	Fresh kidney§§	Brain	Beef heart‖‖	Coagulated egg white	
Fusobacterium (S) type I	Pyorrhea	—	—	A	—	—	—	A	—	—	—	—	—	—	6.4	5.9	+++	7.1	—	—	—	Foul	++	—±	—	+	—	—	—	—	—	—	—	—	—	—	—
Fusobacterium (MiB; A) type II	Pulmonary abscess	—	—	A	—	—	—	A	—	—	—	—	—	—	6.5	5.9	++	7.1	—	—	—	"	++	—	—	+	—	+	—	—	—	—	—	—	—	—	—
Fusobacterium (MiB; B) type II	" "	—	—	A	—	—	—	A	—	—	—	—	—	—	6.5	5.9	++	7.1	—	—	—	"	Few colonies	—	—	+	—	—	Slight A coag.	—	—	—	—	—	—	—	—
Fusobacterium (R) type III	Pyorrhea	—	—	A	—	—	—	A	—	—	—	—	A	—	6.6	6.0	+++	7.1	—	—	—	"	" "	—	—	+—	Ft. tr.	—	—	—	—	—	—	—	—	—	—

* Eckford's method (3) employing iodine as indicator; ++++ complete disappearance of blue color; +++ and ++ partial hydrolysis, reddish-brown color; —no hydrolysis, blue color.

† Bile was incorporated in blood or serum agar plates.

‡ Tested with vanillin and Ehrlich's reagents.

§ Tested for nitrites only.

‖ Cultures grown on lead acetate agar.

** Brom-thymol blue added as indicator.

†† Hemoglobin digested; broth clear; flaky sediment at bottom of tube.

‡‡ Fibrin digested; same appearance as in defibrinated blood.

§§ No digestion; black flakes on surface of kidney; tested after 1 month's incubation.

‖‖ Ground and cooked.

*** Tested with human fibrin and thrombin; method of Tillett and Garner (4).

Types refer to classification of Slanetz and Rettger (1).

A, acid.

Ft. tr., faint trace.

A METHOD FOR DETERMINING THE DIFFERENTIAL SEDIMENTATION OF PROTEINS IN THE HIGH SPEED CONCENTRATION CENTRIFUGE

BY THOMAS P. HUGHES, PH.D., EDWARD G. PICKELS, PH.D., AND FRANK L. HORSFALL, JR., M.D.

(*From the Laboratories of the International Health Division, The Rockefeller Foundation, New York*)

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Optical methods have been devised by Svedberg (1) and Lamm (2) for measuring the sedimentation rate of molecular systems in an intense centrifugal field. These methods utilize either the absorption or refraction of light and, in all cases where they can be applied, offer the advantages of a precise physical measurement. Tiselius, Pedersen, and Svedberg (3) have recently described an ingenious arrangement whereby a photographically recorded sedimentation boundary can be correlated with chemical or biological analysis when the requisite tests can be performed on a quantity of fluid considerably less than 1 cc.

However, the majority of biological laboratories do not possess the apparatus necessary for obtaining and analyzing the photographic records on which the actual sedimentation measurements must be based. Furthermore, many materials of great biological interest, such as hormones, toxins, and viruses, normally occur in such minute concentrations and in fluids of such complex composition that they can be identified, or their concentration estimated, only by biological assay. This evaluation, usually based on serial dilution, requires more fluid than can be contained in the comparatively small cell of the ultracentrifuge. Consequently, an investigation has been made to determine whether reproducible measurements of the sedimentation of small particles can be made with a high speed vacuum centrifuge capable of accommodating relatively large amounts of fluid. The purpose of this paper is to present the results of this study and to describe the methods used, which are based on the chemical analysis or biological assay of material obtained at different fluid levels in the tubular containers after centrifugation.

flasks containing 0.1 per cent cysteine hydrochloride in extract broth. Several pieces of filter paper were introduced in order to provide an adsorbing surface for the bacteria. Immediately before inoculation, the flasks were boiled to drive out oxygen and quickly cooled to 37°C. by means of an ice water bath. The volumes were adjusted to reach the middle of the neck of the flask and covered with a layer of 2 cm. of sterile yellow vaseline. Four or five flasks, containing 24 to 48 hour cultures, were used as seed material with which to inoculate 10 or more flasks of 1.5 to 2.0 liter capacity. The latter were also boiled, cooled, and covered with vaseline. After 2 or 3 days' growth the purity of each mass culture was checked by plating anaerobically and aerobically. The bacteria were thrown down in a Sharples centrifuge and used for the extraction of the protein fractions.

Preparation and Chemical Properties of Bacterial Proteins

The bacterial bodies from about 40 liters of broth were suspended in physiological saline, washed by centrifugation, dehydrated and defatted by means of acetone, ether, and chloroform, dried overnight in a vacuum desiccator, and ground in a Krueger (7) grinding apparatus for 1 or more days until the Gram stain showed complete absence of intact cells. The procedure followed was similar to that described by Heidelberger and Kendall (8) for the preparation of fraction D of *Streptococcus hemolyticus* and included the removal of the unbound carbohydrate and extraction of the bacterial proteins with a phosphate buffer solution at pH 6.5. A brine centrifuge was not available.

Table II summarizes the results of the chemical analyses of four bacterial proteins. The total Kjeldahl nitrogen, after correction for ash, varies from 11.06 to 16.25 per cent. The phosphorus content is close to 1 per cent and varies from 0.87 to 1.33 per cent. The usual color reaction for proteins, the biuret, xanthoproteic, Millon, Hopkins-Cole, and ninhydrin, were positive in all cases. The tests for carbohydrates were as follows: fusobacterium type II gave a positive Molisch reaction; type I was negative; and type III was doubtful. The Bial-Tollens test for pentose was negative for type III, strongly positive for type II, and weakly positive for the type I proteins. Whether or not these carbohydrates are chemically combined with the bacterial proteins and have antigenic or haptene properties, as is the case with the scarlatinal streptococcus studied by Heidelberger and Kendall (9), remains to be determined.

The proteins were digested slowly by pepsin at pH 3.0 and trypsin at pH 8.0 (Table III), when tested by the methods of Anson and Mirsky (10, 11). After digestion they no longer functioned as precipitinogens when mixed with either antibacterial or antiprotein

Methods

The centrifuge used is the air-driven concentration centrifuge of the vacuum type described by Bauer and Pickels (4). It is provided with sixteen celluloid tubes 13 mm. in diameter, oriented at 45° to the axis of rotation. The total fluid capacity is 120 cc. A speed of 27,300 R.P.M. was employed as routine in the present study. The centrifugal force at the different levels of fluid at this speed varied from a minimum of 41,000 to a maximum of 78,000 times gravity, giving an approximate average of 60,000 times gravity.

To permit the removal of centrifuged samples from different levels of the containers without serious stirring or mixing, the instrument shown in Fig. 1 was designed. It consists essentially of a hollow plunger (*a*), a thin cylindrical tube (*b*), a holder (*c*) for the centrifuge tube, and an adjustable stage (*d*) whose vertical movement can be controlled by a rack and pinion (*e*). Both the plunger and the cylindrical tube, which are made of duralumin, have a wall thickness of 0.4 mm., and are open at the top. The bottom end of each is provided with four sector-shaped openings (*f*), which are made as large as possible to minimize capillary action. The plunger is held in a fixed position and is marked with a scale (*g*) so that the depth to which the plunger is inserted into the tube can be measured as the stage is raised. A limited rotary movement of the inner cylindrical tube permits the two adjacent sets of openings to act as a valve.

The hydrogen ion concentration of the solutions studied was measured by the use of the Beckman glass electrode assembly. Relative viscosities were determined with an Ostwald viscosity pipette. Specific gravity was determined pyknometrically.

Chemical analyses for albumin and globulin were done by the method of Howe (5), using 22 per cent Na_2SO_4 at 38°C. to precipitate the globulins and 5 per cent trichloracetic acid for total protein precipitation. The protein-nitrogen content of the various fractions was determined by the method of Van Slyke (6) using sulfuric-phosphoric acid digestion with selenium as a catalyst and oxidation by potassium persulfate. The resulting ammonia-nitrogen was estimated gasometrically. Oxyhemoglobin was determined spectrophotometrically, the optical density of the solutions being determined for light with a wave length of 5412 Å., at which oxyhemoglobin has its maximum absorption in the visible spectrum. The density was correlated with the protein-nitrogen content by repeated micro Kjeldahl analyses.

Experiments were conducted according to the following procedure: 7 cc. of the material to be tested was introduced into each of several celluloid centrifuge tubes. This material was centrifuged at room temperature for a period of 4 hours, comprising an acceleration period of approximately 30 minutes, 3 hours at 27,300 R.P.M., and a deceleration time of approximately 30 minutes. The tubes were then removed from the centrifuge with a minimum of agitation and placed, one at a time, in the sampling instrument described above. The valve on the plunger was opened and the celluloid tube raised until the tip of the plunger just contacted

TABLE III

Disappearance of Precipitin Titers After 6 Days' Digestion of Bacterial Proteins with Purified Pepsin and Trypsin

Immune sera		Precipitin tests: Before proteolysis	Precipitin tests: After proteolysis	Controls: boiled* enzyme	Tyrosine liberated during proteolysis of 5 cc. of 2 per cent solution of protein†
Protein: fusiformis Type I; Enzyme: trypsin‡					
Fusiformis type I (homologous tests)	Antiprotein	1:32,000 ++ 1:16,000 +++	Neg.	8,000 ++	40γ
	Antibacterial	1:8,000 +++	"	8,000 ++	
Fusiformis type II (heterologous tests)	Antiprotein	Neg.	"	Neg.	
	Antibacterial	"	"	"	
Protein: fusiformis Type II; Enzyme: pepsin					
Fusiformis type II (homologous tests)	Antiprotein	1:32,000 ++++ 1:64,000 ++	Neg.	32,000 ++	39γ
	Antibacterial	1:8,000 +++ 1:16,000 ++	"	Neg.	
Fusiformis type I (heterologous tests)	Antiprotein	Neg.	"	"	
	Antibacterial	"	"	"	

* Boiling did not always serve to inactivate these purified enzymes.

† Methods of Anson and Mirsky (10, 11).

‡ Enzymes were purified by the method of Anson and Mirsky and Northrop (12).

TABLE IV

Precipitin Titers of Various Antiprotein Sera Tested with Homologous and Heterologous Fusobacterial Proteins

Antiprotein sera	Bacterial proteins: Fusobacterium I (S)	Fusobacterium II	Fusobacterium III
Fusobacterium (S) type I	++ 32,000	–	–
Fusobacterium type II	–	++ 64,000	–
Streptococcus hemolyticus (group A) (Mic)	–	–	–
Pneumococcus III	–	–	–
Staphylococcus aureus (Mic)	–	–	–

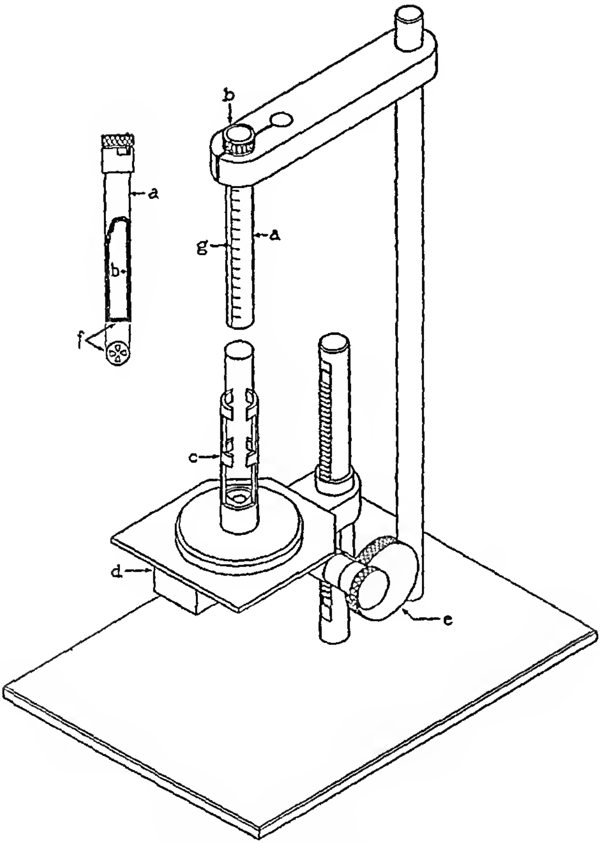

FIG. 1. Details of the sampling apparatus.

TABLE V

Precipitation of Fusobacterial Proteins in the Presence of Unabsorbed Homologous and Heterologous Antibacterial Rabbit Sera

Proteins	Sera										
	Fusobacterium type I	Fusobacterium ((A)-MiB) type II	Fusobacterium ((B)-MiB) type II	Fusobacterium type III	*Strep. evolutus* (NiB)*	*Strep. hemolyticus* (Mic) group A	*Strep. hemolyticus* group A (Lancefield)	*Strep. micros* (Mic N1)*	*Bact. melaninogenicum* (py)*	*Bact. melaninogenicum* (M 19)	*Staph. aureus* (Mic)
Fusobacterium (S) type I	†136,000 ++ 68,000 +++	–	–	–	8,000 ++	34,000 ++++	–	–	–	–	–
Fusobacterium (A (MiB)) type II	–	8,000 ++	16,000 ++	–	–	–	–	–	–	–	–
Fusobacterium (B (MiB)) type II	–	4,000 ++	34,000 ++	–	–	–	–	–	–	–	–
Fusobacterium (R) type III	–	–	–	32,000 ++ 16,000 ++++	32,000 ++	–	–	–	–	–	–

* For additional studies on these bacteria see Weiss and Mercado (13 *a*, *b*).

† Figures refer to maximum dilutions of proteins at which strong precipitation was obtained (++ to ++++). All protein antigen dilutions were analyzed for N and corrections made for undissolved residues.

the top of the fluid column. After the scale reading had been noted, the tube was raised exactly 1 cm. and the valve closed, thus trapping a measured sample of liquid within the plunger. A pipette was introduced into the closed chamber of the plunger and the fluid contained therein removed. The valve was again opened and the tube raised another centimeter and the sampling repeated. In all, five such samples were taken. The remaining fluid was mixed with a pipette to resuspend any sediment and transferred to a suitable tube. The plunger was carefully cleaned and dried. The remaining tubes were similarly sampled. The

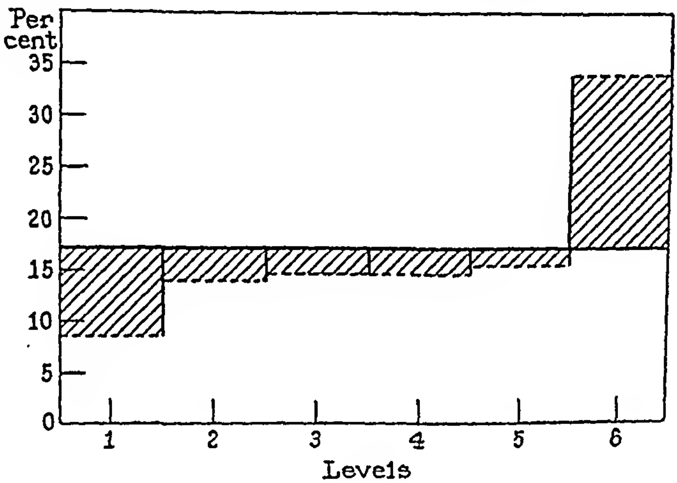

Fig. 2. Illustrative diagram of sedimentation factor. The solid horizontal line indicates the percentage of the total globulin initially present at each of the six levels. The levels of the centrifuge tube are numbered from the top downward. Each broken line indicates the percentage of the total globulin which is present at the corresponding level following centrifugation. Each section of the shaded area represents the difference between the initial and final percentage values for a particular level. The total shaded area corresponds to the sedimentation factor

fluid from corresponding levels of the several tubes was pooled and samples taken for chemical analysis or biological titration.

With this method it has been found possible to sample a fluid at any desired number of levels in a comparatively short time and with practically no stirring.

EXPERIMENTAL

Calculation of Results.—To express the degree of sedimentation the term sedimentation factor (S.F.) has been adopted as a basis for comparing results. Its definition and use will be made clear by reviewing its application in the following experiment.

tinued this work, concluded that this so called P substance was really a colloidal salt of an inorganic acid and not a protein, since it was resistant to proteolytic enzymes, including pepsin and trypsin. Subsequent investigations by Ivánovics and Bruckner (19) demonstrated that the haptene of the anthrax bacillus and of *Bacillus mesentericus* was in the nature of a polypeptide containing *l*-glutamic acid.

Our preliminary data indicate that there is also a group specific carbohydrate among the fusobacteria, which is extractable by the methods of Heidelberger and Avery (20). The presence of foul and poisonous gases in mass cultures of the fusobacteria has, however, retarded this work which is still in progress.

The demonstration of type specific proteins among the fusobacteria will, it is hoped, make possible further work on the serological classification of this interesting group of organisms, permitting precipitin tests to take the place of the uncertain and irregular agglutination tests. As shown by Varney (21), and Slanetz and Rettger (1), spontaneous agglutination occurs frequently with these organisms. These data may also aid in the study of the problem of the pathogenicity of the various fusobacteria and their possible rôle in the etiology of pulmonary abscess, bronchiectasis, Vincent's angina, pyorrhea alveolaris, etc. (22, 23).

SUMMARY AND CONCLUSIONS

1. The classification of fusobacteria into types, as suggested by Slanetz and Rettger on the basis of their cultural and biochemical characteristics, was confirmed. The following additional data are presented: the behavior of types I, II, and III in several previously untested carbohydrates; the final pH in 1 per cent glucose broth; the hydrolysis of starch; the failure to decompose sodium hippurate and citrates; the absence of pathogenicity for several types of experimental animals; and the absence of fibrinolysin and of proteolytic enzymes.

2. By the use of a technic described in the text, it was possible to grow the anaerobic fusobacteria in mass cultures.

3. With the methods of Heidelberger and Kendall, immunologically type specific proteins were extracted from fusobacteria types I, II, and III. This observation, which is in a sense an extension of the

Normal horse serum was diluted 1:5 with 0.9 per cent NaCl solution. The original specific viscosity of the diluted serum was found to be 1.095; its specific gravity, 1.018; and pH, 7.6. The material was centrifuged for 3 hours at a speed of 27,300 R.P.M. Then samples were taken at six different levels for chemical analysis. The results are shown in Fig. 2 and Table I. In uncentrifuged serum the globulin would be equally distributed among the six fractions, each containing one-sixth or 16.66 per cent of the total amount, as represented by the solid line. The broken lines represent the percentages of globulin actually found in each fraction after centrifugation. The sedimentation factor is represented by the shaded region, which is specifically the arithmetic sum of the changes in the per

TABLE I

Sedimentation of Globulin in Diluted Serum

Level	A Globulin-nitrogen	B Total globulin in fraction	C Sedimentation factor (S.F.)
	mg. per cc.	*per cent*	
1	0.747	8.43	8.23
2	1.210	13.68	2.98
3	1.278	14.44	2.22
4	1.297	14.66	2.00
5	1.339	15.12	1.54
6	2.980	33.67	17.01
Total............	8.851	100.00	33.98

cent values which have been produced in the several samples by centrifugation. These values are given in Table I.

The fractions are numbered from the top of the tube downward. Column A indicates the result of the chemical analysis. The sum of these values, 8.851 mg., may be considered as the total amount of globulin-nitrogen present, since the volumes of all the samples are equal. Column B is the percentage of the total globulin present in each of the fractions, and is obtained by dividing the value given in column A by the total amount. Column C is the arithmetic difference between 16.66 and the value given in column B. The sum of these differences is the total sedimentation factor, in this case 33.98.

It should be noted that in the event of no sedimentation, the sedimentation factor becomes zero, and that in the event of complete sedimentation, the factor reaches its maximum value of approximately 167, in which case all the material is contained in the bottom sample.

The same method of calculation can be employed by substituting biological assay for chemical analysis, as will be indicated subsequently.

Reproducibility of Technique

The reproducibility of results was tested in two ways: (*a*) by comparing the analyses of separate tubes of identical material centrifuged in the same run and (*b*) by comparison of results obtained on separate runs under similar conditions, with identical preparations of material. The results obtained in the first case should evaluate the sampling technique, while those obtained in the second case would indicate the effectiveness of the speed control of the centrifuge, the agitation during deceleration, and other factors connected with the instrument itself. The data obtained for this purpose are presented in the following sections.

Reproducibility of Sampling Technique.—To test the reproducibility of the sampling technique a preparation of oxyhemoglobin was used.

The hemoglobin was prepared from monkey blood by repeated washing of the cells with 0.9 per cent NaCl solution, liberation of the hemoglobin by laking the cells by suspension in water, and, finally, clarification by filtration through a Seitz filter. 7 cc. of this solution was introduced into each of sixteen celluloid centrifuge tubes, and centrifuged for 3 hours at 27,300 R.P.M. The original specific viscosity was 1.011; the specific gravity was 1.002; and the pH was 6.95. After centrifugation the tubes were divided into four lots of four tubes each. Samples were taken as previously described, and those from corresponding levels of each of the four tubes in a lot were pooled. Thus material was provided for four duplicate sampling and analysis procedures.

The results as presented in Table II indicate that the sampling technique is reproducible to within 6 per cent. The average variation of the total sedimentation factor from the mean is 2.9 per cent. These results were obtained when using oxyhemoglobin, a protein of very small particle size and, consequently, difficult to sediment. Furthermore, the solution was quite dilute, giving ideal conditions for inadvertent remixing. Accuracy would, presumably, be increased with an increase in concentration or in particle size.

Reproducibility of Centrifugation.—The reproducibility of results between subsequent runs was next tested as follows:

A 10 per cent solution of normal monkey serum was made in 0.9 per cent NaCl solution and divided into four lots. The original specific viscosity was 1.076; the specific gravity was 1.010; and the pH was 7.80. Each lot was centrifuged separately for 3 hours at a speed of 27,300 R.P.M. Samples were taken at different

at room or refrigerator temperature. Autopsy or excised syphilitic tissue has been shown to be infective after 24 hours by Lacy and Haythorne (2), after 48 hours by Gregoriev and Jarysheva (3), and after 5 days by Schaffer (4). Recently Rosahn (5), working with excised syphiloma of rabbits' testis which was kept at refrigerator temperature, observed typical lesions of experimental syphilis in rabbits inoculated with 48 hour specimens, but 96 hour specimens produced only slight lesions in 1 of 3 animals. Neither of 2 rabbits inoculated with 7 day specimens exhibited lesions but 1 was infected as proved by lymph node transfer. Neither of the 2 animals inoculated with 14 day, 23 day, and 42 day specimens developed syphilitic lesions, and lymph node transfer was negative in each.

Cultivation of treponemes on artificial media has also failed to provide a satisfactory method for maintaining or propagating organisms which are pathogenic for animals. In a recent review of the subject Kast and Kolmer (6) point out that only in rare instances have treponemes maintained in culture been shown to be virulent for rabbits. These authors likewise failed to grow *T. pallidum* in tissue culture. Essentially the same statements would seem to be applicable to *T. pertenue*.

The Method of Freezing

During the course of attempts to freeze and desiccate treponemes by the technique commonly employed for certain bacteria and filtrable viruses, it was observed that although the treponemes did not ordinarily survive desiccation they were apparently unharmed by the freezing process alone. With this observation as a basis a very simple technique for preserving the virulence of treponemes *in vitro* was devised.

Solid carbon dioxide (dry ice) when placed in 95 per cent ethyl alcohol reduces the temperature of the liquid medium to approximately −78°C., which temperature is above the freezing point of alcohol. This temperature is maintained as long as dry ice is present. When tissue is immersed in this medium, it freezes solid within a few seconds. Any well insulated container may be used for the dry ice-alcohol mixture, but a 12 gallon size container, insulated by vacuum, has been found to be most convenient.[1] Such a container will accommodate about 50 pounds of dry ice, 6 to 8 gallons of alcohol, and still leave space for many specimens. There is little or no evaporation of alcohol. Dry ice must be replenished every 5 to 7 days, approximately 70 pounds per week affording a safe margin. The top of the container is allowed to fit snugly but it is left unsealed in order to permit the escape of gas. The container is kept at room temperature.

The material to be preserved may be frozen as small pieces of tissue or as a

[1] The container used in these experiments was obtained from the American Thermos Bottle Company, Norwich, Connecticut.

levels in the manner described above, and a quantitative determination of albumin was made. The results of the four different runs on the same material are given in Table III.

TABLE II

Sedimentation of Oxyhemoglobin in Four Identical Samples Centrifuged Simultaneously

Level	Sample 1			Sample 2			Sample 3			Sample 4		
	mg. per cc.	*per cent*	*S.F.*	*mg. per cc.*	*per cent*	*S.F.*	*mg. per cc.*	*per cent*	*S.F.*	*mg. per cc.*	*per cent*	*S.F.*
1	0.440	13.18	3.48	0.452	13.08	3.58	0.460	13.32	3.34	0.456	13.24	3.42
2	0.445	13.33	3.33	0.480	13.88	2.78	0.460	13.32	3.34	0.468	13.59	3.07
3	0.456	13.66	3.00	0.480	13.88	2.78	0.484	14.02	2.64	0.495	14.37	2.29
4	0.503	15.07	1.59	0.515	14.90	1.76	0.516	14.94	1.72	0.519	15.07	1.59
5	0.519	15.55	1.11	0.554	16.03	0.63	0.558	16.16	0.50	0.530	15.39	1.27
6	0.975	29.20	12.54	0.975	28.21	11.55	0.975	28.24	11.58	0.975	28.31	11.65
Total......			25.05			23.08			23.12			23.29
Variation from mean, *per cent*..................			6.0			2.3			2.2			1.4

TABLE III

Sedimentation of Albumin during Four Separate Centrifugations on the Same Lot of Diluted Monkey Serum

Level	Run 1			Run 2			Run 3			Run 4		
	mg. per cc.	*per cent*	*S.F.*	*mg. per cc.*	*per cent*	*S.F.*	*mg. per cc.*	*per cent*	*S.F.*	*mg. per cc.*	*per cent*	*S.F.*
1	0.553	10.26	6.40	0.508	9.40	7.26	0.588	8.81	7.85	0.476	8.67	7.99
2	0.790	14.66	2.00	0.752	13.91	2.75	1.014	15.19	1.47	0.706	12.87	3.79
3	0.789	14.64	2.02	0.798	14.76	1.90	1.014	15.19	1.47	0.860	15.68	0.98
4	0.866	16.07	0.59	0.818	16.13	0.53	1.120	16.78	0.12	0.950	17.32	0.66
5	0.973	18.06	1.40	0.998	18.46	1.80	1.120	16.78	0.12	0.952	17.35	0.69
6	1.416	26.28	9.62	1.532	28.33	11.67	1.818	27.24	10.58	1.540	28.33	11.67
Total........			22.03			25.90			21.61			25.78
Variation from mean, *per cent*..................			7.8			8.7			9.3			8.2

The reproducibility between runs does not appear to be as good as that of sampling. The maximum variation between these runs is under 10 per cent, and the average variation from the mean is 8.5

frequent intervals. A positive result was recorded only when the animal presented a lesion characteristic of the one or the other experimental disease and actively motile treponemes were demonstrated in the lesion. In two instances the source material was first emulsified in physiological saline and the resulting suspension used for virulence tests before and after freezing. Thus while the dose for most specimens was only approximately the same at different intervals, in these two specimens (rabbits 2-46 and 2-45) the individual dose for each inoculation was exactly the same (0.3 cc.).

Test inoculations were commonly made at intervals of 14 days, 1 month, 2 months, 4 months, and 6 months, after the material had been frozen, and the virulence of several specimens was tested after 1 year. The first tests made with both syphilis and yaws treponemes may be considered preliminary in nature for the technique as now employed was not then fully developed. Thus in some instances only a few portions of the same specimen were frozen and in several instances a virulence test before freezing was not made. The results with the earlier specimens were not as uniform as with the later specimens.

Effect of Storage at −78°C. Temperature on Morphology and Motility.—When material containing treponemes is withdrawn from the freezing mixture and warmed, thawing is complete within about 5 minutes. A dark field examination at this time shows the treponemes to be normal in shape and size but usually inactive. As the slide is warmed by the heat from the dark field light the spirochetes begin to move, sluggishly at first, but within a few minutes many will exhibit very active motility. In most of the specimens given in Table I the proportion of active treponemes and the degree of motility did not seem to vary significantly, after various periods of time, from that observed before freezing. In some frozen specimens the proportion of active treponemes seemed to be less than before freezing and in a few instances only an occasional treponeme was found to be motile. Frozen specimens from the same lesion, removed from the freezing mixture at different times, occasionally showed variation in motility of the treponemes but there was no observable relation to the duration of the frozen state. Specimens showing poor motility frequently retained their virulence, however, and it is possible that the diminution in motility may not have been an expression of any deleterious effect of the freezing mixture but may have been due to other causes, such as variation in the temperatures to which the treponemes were subjected after thawing. With the exception of the first specimens frozen (Nos. 1-65 and 1-68) all of those examined after 6 months and 1 year showed enormous numbers of actively motile treponemes.

per cent. Here again a small particle-sized protein in dilute solution is used as a test material. The cause of this variation is not obvious, but may be partially accounted for by slight variations in the speed of the centrifuge or by an agitation produced by deceleration.

Partial Separation of Proteins

To illustrate the separation of proteins as measured by sedimentation factors the results of three tests are presented: (*a*) the separate but simultaneous centrifugation of ovalbumin and limulus hemocyanin, (*b*) the relative sedimentation of serum albumin and serum globulin in the same sample of serum, and (*c*) the relative sedimentation of serum protein and yellow fever virus contained in the same serum sample.

TABLE IV

Sedimentation of Ovalbumin and Hemocyanin Centrifuged Simultaneously

Level	Hemocyanin			Ovalbumin		
	mg. per cc.	*per cent*	*S.F.*	*mg. per cc.*	*per cent*	*S.F.*
1	0.017	0.54	16.12	2.298	14.05	2.61
2	0.016	0.51	16.15	2.566	15.69	0.97
3	0.025	0.81	15.85	2.619	16.01	0.65
4	0.028	0.90	15.76	2.610	15.96	0.70
5	0.023	0.75	15.91	2.767	16.92	0.30
6	2.986	96.47	79.81	3.491	22.74	6.38
Total....			159.60			11.61

Comparative Sedimentation of Ovalbumin and Hemocyanin.—Ovalbumin was prepared by crystallization of the albumin from eggs by the use of ammonium sulfate and acetic acid. The protein was twice recrystallized and finally dissolved in water. The pH was 6.65; the original specific viscosity was 1.014; and the specific gravity was 1.002. Hemocyanin was prepared from the blood of *Limulus polyphemus* by the method of Redfield, Coolidge, and Shotts (7). This protein was finally dissolved in 1.0 per cent NaCl solution and made slightly alkaline to prevent precipitation. The pH of the solution was 8.0; the specific viscosity, 1.093; and the specific gravity, 1.023. Four tubes of each of the two preparations were centrifuged simultaneously, then sampled and pooled as previously described. The results obtained on chemical analysis of these samples are given in Table IV.

The S.F. value obtained for hemocyanin is 159.60, while that for ovalbumin is 11.61. It seems apparent, therefore, that this method,

TABLE I

Infectivity for Rabbits of Treponema pallidum and Treponema pertenue Which Had Been Maintained at Temperature of −78°C. for Varying Periods up to 1 Year

	Source of treponemes Rabbit No.	Result of virulence test before freezing*	Results of virulence tests at different intervals after freezing*						
			14 days	21 days	1 mo.	2 mos.	4 mos.	6 mos.	1 yr.
Treponema pallidum	1-65	Pos. 17	Pos. 21	Pos. 17	Pos. 21	Pos. 57	Pos. 46	Pos. 33	— —
	1-66	Pos. 17	Pos. 27	Pos. 21	— —	— —	— —	— —	— —
	2-21	— —	Pos. 36	— —	— —	Pos. 41	Pos. 40	— —	— —
	2-23	— —	Pos. 30	— —	Pos. 31	Neg. —	— —	— —	— —
	2-33	Pos. 8	Pos. 25	— —	Pos. 19	Pos. 12	Pos. 7	Pos. 16	Pos. †‡
									Pos. 22
	2-37	Pos. 8	Pos. 9	— —	Pos. 10	Pos. 17	Pos. 21	Pos. 13	Pos.‡ 9
								Pos. 21	Pos.‡ 9
	2-39	Pos. 12	Pos. 17	— —	Pos. 14	Pos. 7	Pos. 8	Pos. 27	— —
	2-46	Pos. 16	Pos. 32	— —	Pos. 22	Pos. 22	Pos. 18	Pos. 23	Pos.‡ 22
								Pos. 24	Pos. 33
Treponema pertenue	1-68	Pos. 28	Pos. 7	Pos. 21	Pos. 27	Pos. 30	Quest.§ 40	Pos. 35	Quest.§ 40
								Pos. 28	— —
	1-70	Pos. 14	Pos. 18	Pos. 26	— —	— —	— —	— —	— —
	2-20	— —	Pos. 41	— —	Pos. 29	Pos. 30	Pos. 42	— —	— —
	2-27	Pos. 17	Pos. 19	— —	Pos. 17	Pos. 16	Pos. 14	Pos. 7	— —
	2-29	Pos. 22	Pos. 22	— —	Pos. 20	Pos. 10	Pos. 21	Pos. 23	— —
	2-45	Pos. 28	Pos. 24	— —	Pos. 24	Pos. 28	Pos. 20	Pos. 21	— —
	2-68	— —	— —	— —	— —	— —	— —	— —	Pos. 25
	2-69	— —	— —	— —	— —	— —	— —	— —	Pos. 19

* Numerals denote incubation period in days.
† Clinical lesion masked by non-specific reaction. Motile *T. pallidum* demonstrated, 33 days.
‡ Rabbit developed metastatic orchitis and multiple generalized lesions.
§ Questionable result. Clinical lesion but *T. pertenue* not demonstrated.

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with our standard speed of 27,300 R.P.M., will give a characteristic value for even the smallest proteins. According to Svedberg (1), the molecular weight of ovalbumin is approximately 35,400, while that of the heavier component of limulus hemocyanin is 3,000,000. In this experiment practically all the hemocyanin was concentrated in the bottom sample, and a more characteristic value for its sedimentation factor would be obtained by centrifuging for a shorter length of time.

Relative Sedimentation of Albumin and Globulin in Serum.—To illustrate the separate sedimentation of two components in the same solution duplicate runs of monkey serum were made. The fractions were analyzed for serum albumin and

TABLE V

Sedimentation of Albumin and Globulin in Two Diluted Sera Centrifuged Separately

Level	Serum 1						Serum 2					
	Albumin			Globulin			Albumin			Globulin		
	mg. per cc.	*per cent*	*S.F.*	*mg. per cc.*	*per cent*	*S.F.*	*mg. per cc.*	*per cent*	*S.F.*	*mg. per cc.*	*per cent*	*S.F.*
1	0.553	10.26	6.40	0.747	8.44	8.22	0.588	8.81	7.85	0.464	7.58	9.08
2	0.790	14.66	2.00	1.210	13.67	2.99	1.014	15.19	1.47	0.776	12.67	3.99
3	0.789	14.64	2.02	1.228	14.43	2.23	1.014	15.19	1.47	0.894	14.60	2.06
4	0.866	16.07	0.59	1.297	14.65	2.01	1.120	16.78	0.12	0.876	14.30	2.36
5	0.973	18.06	1.40	1.339	15.13	1.53	1.120	16.78	0.12	1.076	17.57	0.91
6	1.416	26.28	9.62	2.980	33.67	17.01	1.818	27.24	10.58	2.038	33.28	16.62
Total...			22.00			34.00			21.60			35.00

serum globulin separately. Two separate monkey sera were diluted to a 10 per cent concentration in 1 per cent NaCl solution. Serum 1 bad an original specific viscosity of 1.052, a specific gravity of 1.092, and a pH of 7.88. Serum 2 had an original specific viscosity of 1.076, a specific gravity of 1.009, and n pH of 7.80. Centrifugation in each case was for 3 hours at 27,300 R.P.M. Samples were taken in the usual manner and analyzed separately for albumin and globulin-nitrogen. The results are given in Table V.

The ratio of the sedimentation factor of albumin to that of globulin in serum 1 is 0.65, while with serum 2 it is 0.62. The values obtained for the two albumin fractions are in close agreement, as are those obtained for the globulins.

Relative Sedimentation of Yellow Fever Virus and Serum Proteins.—To demonstrate the application of biological titration to this method the data obtained by centrifuging a suspension of yellow fever virus in monkey serum are now presented.

fresh material from the same source. In one instance the inoculation of fresh material failed to produce a lesion while inoculation of the same amount of material from the specimen 2 months after freezing gave rise to typical lesions of experimental yaws. This experiment indicates, therefore, that probably all strains of yaws and syphilis spirochetes will survive freezing at −78°C. Moreover, the infectivity of material containing relatively few spirochetes, as well as of material containing large numbers of these organisms, is preserved.

TABLE II

Virulence for Rabbits of Various Strains of Syphilis and Yaws Spirochetes Which Had Been Maintained at −78°C. for 2 Months

Syphilis material, popliteal nodes				Yaws material, testicular emulsion			
Strain of treponemes	Rabbit No.	Virulence test before freezing*	Virulence test 2 mos. after freezing*	Strain of treponemes	Rabbit No.	Virulence test before freezing*	Virulence test 2 mos. after freezing*
S-4	1-80	Pos. 31	Pos. 39	YB	2-31	Pos. 40	Pos. 35
S-5	1-83	Pos. 25	Pos. 33	YC	1-52	Pos. 32	Pos. 36
S-6	1-84	Pos. 13	Pos. 33	YD	1-75	Pos. 37	Quest.† 41
S-8	1-86	Pos. 25	Pos. 36	YE	1-28	Neg.	Pos. 43
S-10	1-89	Pos. 25	Pos. 27	YF	2-43	Pos. 45	Pos. 31
				YH	1-59	Pos. 32	Pos. 60
				YK	2-24	Pos. 34	Pos. 30

* Numerals denote incubation period in days.

† Questionable result. Clinical lesion but *T. pertenue* not demonstrated.

Effect of Other Freezing Temperatures on Treponemes.—Specimens of rabbits' testes rich in treponemes were prepared as in the above experiments. The material in sealed tubes was frozen rapidly at −78°C. and after 2 hours transferred to the ice-making compartment or tray of an ordinary electrically operated refrigerator. By immersing the tubes in 95 per cent alcohol, the tissue was maintained in the frozen state at a temperature approximating −10°C. It was soon evident that treponemes survived at this temperature for much shorter periods than they did at −78°C. and no attempt was made to determine accurately their survival time. The following observation, however, may be noted.

The virus suspension was prepared by grinding the brain
an experimentally induced yellow fever encephalitis in a
of normal monkey serum in 1 per cent NaCl solution.
sion was prepared and centrifuged at 3000 R.P.M. to se
tissue, and the supernatant fluid was then passed thr
filtrate was centrifuged at 27,300 R.P.M. for 3 hours, and
manner. The protein content of each fraction was d
the virus content titrated by the serial decimal dilutio
inoculation of each dilution intracerebrally into a gro
of the virus suspension is calculated by the method of R
results obtained are presented in Table VI.

TABLE VI

Sedimentation of Yellow Fever Virus and

Level	Serum protein			
	mg. per cc.	*per cent*	*S.F.*	*lethal units*
1	0.526	8.22	8.44	
2	0.895	13.99	2.67	
3	0.954	14.91	1.75	
4	0.998	15.59	1.07	1
5	1.098	17.16	0.50	
6	1.928	30.13	13.47	72,21
Total.....			27.90	

While the virus has been completely sedime
for the whole serum is about midway between
the separate estimations of albumin and gl
These results are

was frozen at −78°C. and maintained at this temperature for 2 months. Another lot was frozen at −78°C. and after 2 hours transferred to an electrically operated freezing unit which maintains a temperature of about −20°C. A third lot was frozen at −20°C. and after 2 hours transferred to a maintenance temperature of −78°C., and a fourth lot was frozen and maintained at −20°C. Inoculation of the emulsion before freezing produced a typical syphilitic orchitis within the usual incubation period. 2 months after freezing a specimen from each lot was examined for motility of the treponemes and its virulence was tested by intratesticular inoculation of 2 rabbits with 0.3 cc. of the emulsion. The results of these tests are shown in Table III. In both specimens maintained at −78°C. the treponemes were actively motile and produced typical lesions of experimental syphilis in rabbits. No difference was noted between the specimen frozen at −78°C. and that frozen at −20°C. In neither specimen which was maintained at −20°C. were treponemes motile. The virulence tests on the specimens frozen at −78°C. and maintained at −20°C. were, unfortunately spoiled, but rabbits inoculated with material which had been frozen and maintained at −20°C. remained negative for 90 days.

It seems clear that freezing treponemes at −20°C. does not affect the organisms adversely, but maintenance at this temperature over a period of 2 months causes their death.

Effect of Freezing and Desiccation of Treponemes

Tissue containing many syphilis treponemes from 14 different sources was frozen and dried in a glass desiccator. Examination of the dried material after the addition of water showed that the number of treponemes was considerably less than before freezing, all were non-motile and many were distorted. In 5 instances the infectivity for rabbits of dried material from as many different sources was tested with but one positive result. In each case inoculation of material from the same source before freezing gave rise to typical lesions. The quantity of material inoculated after drying was equivalent to from 5 to 15 times the amount of the control inoculum. 3 rabbits inoculated with material 1 day after desiccation remained negative for 3 months and transfer of their popliteal lymph nodes to 2 other rabbits yielded negative results. 3 specimens inoculated 14 days after drying likewise gave negative results. In 1 instance, however, dried material inoculated the day after desiccation gave rise to typical syphilitic orchitis in each of 2 rabbits, 38 and 45 days, respectively, after inoculation, the lesions being rich in treponemes. The incubation period in the control rabbit was 18 days.

It appears that while occasionally *T. pallidum* may survive freezing and desiccation, the technique as employed does not offer a favorable method for preserving these organisms.

dency to separate from the medium under the action of centrifugal forces. The primary factors now known, aside from the design of the centrifuge rotor, which control the degree of sedimentation under the conditions outlined are: (*a*) the intensity of the centrifugal forces applied, (*b*) the time of centrifugation, (*c*) the difference in specific gravity between the particles and their suspending medium, (*d*) the viscosity of the suspending medium, (*e*) the electrical charges on the particles, and (*f*) the size, shape, and degree of homogeneity of the particles. The first two factors are readily controllable. For most aqueous protein solutions the differential specific gravity will not vary significantly from a value of about 0.33; similarly, unless the concentration of protein or some other dissolved substance is unusually high, the viscosities will be approximately the same. The effect of electrical charge can be minimized by careful adjustment of the pH and the inclusion of an adequate concentration of electrolytes in the suspending medium. Theoretically, sedimentation factors could then be compared as approximate measurements depending only on the size, shape, and homogeneity of the several protein species under investigation. It is not immediately evident from a single experiment whether a gradual, progressive increase in the concentration of material from one fluid level to another is due wholly to diffusion or to an inhomogeneity in the separating particles which causes them to settle at different rates. A series of experiments varying the duration or speed of centrifugation should give information on this point. Obviously, the particle size is of greatest importance in determining sedimentation factors, and rough comparisons can be made on this basis.

Attention should, however, be called to the fact that some factors other than those cited may influence sedimentation as measured by this method. It was found that significant changes occurred in the sedimentation factors of several proteins when more dilute solutions were employed. However, even very wide variations in the concentration do not change the sedimentation factor of the materials studied more than twofold, the sedimentation factor in every case decreasing with the concentration. Other investigators (9–12) have observed a marked dilution factor of a similar nature with some proteins studied in Svedberg's ultracentrifuge. It is hoped that a series of experiments now in progress will establish definitely whether the effect observed in the concentration centrifuge is due wholly to changes associated

The results of the several titrations are shown in Table IV. The number of deaths is shown as the ratio of the number dying of pneumonia to the number surviving on the 3rd day after inoculation. All surviving mice were killed on the 10th day and the number showing lung lesions together with those dying of pneumonia are shown as the ratio to the number surviving on the 3rd day. It will be evident that no appreciable decrease in the virulence of the virus occurred during the period of freezing. With a dilution of 1 to 100,000 some deaths occurred in each titration, while lung lesions were produced

TABLE IV

Effect of Freezing at −78°C. on the Virus of Human Influenza

Dilution of virus	Results of mouse inoculation*							
	Before freezing		After freezing					
			2 mos.		4 mos.		6 mos.	
	Deaths	Lung lesions	Deaths	Lung lesions	Deaths	Lung lesions	Deaths	Lung lesions
10^{-1}	—	—	4/4	4/4	6/6	6/6	—	—
10^{-2}	4/4	4/4	4/4	4/4	6/6	6/6	6/6	6/6
10^{-3}	3/4	4/4	4/4	4/4	5/5	5/5	6/6	6/6
10^{-4}	4/4	4/4	4/4	4/4	4/6	6/6	4/6	5/6
10^{-5}	1/4	4/4	3/4	4/4	4/6	6/6	3/6	6/6
10^{-6}	0/4	4/4	0/4	4/4	0/6	5/6	0/6	5/6
10^{-7}	0/4	0/4	0/4	0/4	0/6	0/6	0/6	0/6

* Ratio of number of mice dying or showing lung lesions to number inoculated. Numerator denotes number dying or showing lung lesions; denominator denotes number inoculated, exclusive of number dying before 3rd day.

quite uniformly by a dilution of 1 to 1,000,000. Several other strains of human influenza virus were maintained at −78°C. for periods of from 3 to 4 months. While no titration of this material was made there was no indication of a decrease in the infectivity for mice of the frozen material. Apparently, the virulence of the virus is maintained as well when whole mouse lungs are frozen as when the emulsified lung tissue is first suspended in saline or broth.

The Virus of Yellow Fever.—The titer of 2 strains of yellow fever virus was determined before freezing and after maintenance at −78°C. for 2 and 6 months.

with the material, or whether the efficiency of the method itself is markedly responsive to changes in concentration of the substance being studied.

An insufficient number of observations are available at present to establish a definite relationship between the sedimentation constant of Svedberg and the sedimentation factor. It is hoped that on further investigation an approximate correlation can be developed between the two measurements. At the present time the sedimentation factor determination gives a simple and direct method of (*a*) determining the relative sedimentation of a protein, (*b*) determining the fraction of a protein mixture with which biological activity is associated, (*c*) testing the purity of biologically active preparations, and (*d*) a general method of investigating the effects of various chemical and physical factors on the sedimentation of particles suspended in a field of high centrifugal force.

SUMMARY

A method is presented for determining the relative degree of sedimentation of proteins and other small particles, either in pure form or in a mixture, utilizing the concentration centrifuge of Bauer and Pickels. Chemical analysis or biological assay of material obtained at different fluid levels by means of a special sampling device is used to measure the degree of sedimentation. The results are reproducible within 10 per cent and, under the conditions of the experiments cited, are characteristic for the proteins thus far investigated.

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The results of titration before freezing and at 2 months and 6 months after freezing are shown in Table V. The titer at each period was essentially the same, namely 10^{-4}.

The Virus of Spontaneous Encephalomyelitis of Mice.—This virus was first described by Theiler (9).

Brains of infected mice were emulsified in plain broth to make a 10 per cent suspension. Several portions of this suspension were frozen at $-78°C$. From the fresh material tenfold serial dilutions were made and titrated by intracerebral inoculation of Swiss mice with 0.03 cc. amounts. The suspension was bacteriologically sterile. The usual incubation period of this virus is somewhat longer than that of yellow fever virus and varies from 6 to 18 days. Paralysis of the extremities frequently supervenes several days before death of the mouse. Young mice seem to be more susceptible than older mice (9). Surviving mice were observed for 21 days; deaths occurring before the 6th day were regarded as nonspecific. The results of titration of the virus before freezing and 2 months and 6 months after freezing are shown in Table V.

While the end-points of the titrations are not as sharp as those obtained with yellow fever virus, it would appear that no material decrease in the titer of this virus occurred over the 6 month period during which it was being maintained at $-78°C$.

DISCUSSION

About the beginning of this century it was shown by Brown and Escombe (13) and by Thiselton-Dyer (14) that certain seeds retained the ability to germinate after exposure to the temperature of liquid air ($-190°C.$) for 110 hours and to the temperature of liquid hydrogen ($-250°C.$) for 6 hours. Macfayden (15) demonstrated that various bacteria, including *Bacillus typhosus*, *Corynebacterium diphtheriae*, and *Vibrio cholerae*, were viable after exposure to these same temperatures for 20 hours. A number of years later a technique for the preservation of various bacteria, filtrable viruses, and antibodies by desiccation from the frozen state was developed through the studies of Shackel (16), Swift (17), Sawyer, Lloyd, and Kitchen (11), Elser, Thomas, and Steffen (18), Flosdorf and Mudd (19), and others. In the earlier studies material to be desiccated was frozen in an ice-salt mixture at a temperature a few degrees below 0°C. and it appears that solid CO_2 was first employed for the freezing process prior to

THE DEMONSTRATION OF LESIONS AND VIRUS IN THE LUNGS OF MICE RECEIVING LARGE INTRAPERITONEAL INOCULATIONS OF EPIDEMIC INFLUENZA VIRUS

By E. R. RICKARD, M.D., and THOMAS FRANCIS, Jr., M.D.

(*From the Laboratories of the International Health Division, The Rockefeller Foundation, New York*)

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In attempting to induce the adaptation of a strain of human influenza virus, PR8 (1), to the central nervous system of mice, large doses of virus suspension were given intraperitoneally to the animals while simultaneous injections of meat infusion broth were made intracerebrally. No external evidence of infection was noted, but when the mice were sacrificed on the 7th day after inoculation pulmonary lesions of moderate extent were observed. The lesions in the gross were quite characteristic of those produced in the lungs of mice by the intranasal inoculation of influenza virus. They consisted of plum colored areas of edematous pneumonia involving one-fourth to one-half the lung tissue. Since no evidence had previously been obtained to suggest that pulmonary infection occurred with influenza virus administered by other than the intranasal route, further studies were undertaken to determine the distribution of virus in the animal body after inoculation by various routes.

While the present work was in progress, Smorodintsev and his coworkers (2) reported certain results showing that influenza virus could be recovered from the blood of mice for a period after its introduction into the peritoneal cavity but observed no pulmonary lesions. The present report is primarily concerned with the localization and survival of influenza virus in various organs of mice, especially the lungs, after its inoculation by the intraperitoneal route.

Materials and Methods

The PR8 strain of epidemic influenza virus maintained in mice through 150 to 240 serial intranasal passages was used throughout. Intranasal inoculation of

gelatin gels found that on slow freezing at $-19°C.$ ice formed largely on the surface of the gel from water which had been extracted from the core of the mass. Subjection of the gel to still lower temperatures caused further movement of water from the core to the surface, until the gel reached a concentration of 65 to 66 per cent. Beyond this point no more water was released even when the gel was subjected to a temperature of $-190°C.$ In other words, a certain portion of the water had been "bound" by the colloid and was, in that state, unfreezable. If the rate of freezing was very rapid ice formed evenly throughout the entire mass of colloid material.

The studies just cited were all directed toward determining the physical changes which take place during the freezing process. While it is evident that certain changes do occur at or soon after the moment of freezing it seems clear that such changes cannot explain the observations made upon the effect of freezing on treponemes. *T. pallidum* survived freezing at $-10°C.$ and $-20°C.$ but died during subsequent days or weeks when maintained at these temperatures. This suggests that the damage to the organism which occurs at these temperatures is not due to physical alterations in the cell protoplasm at the time of freezing but to changes which occur during the maintenance period. It has been suggested by Belehradek (26) that death of cells in the frozen state occurs as a result of the accumulation of the products of metabolism, that the metabolic rate is lower at very low temperatures than at higher temperatures. While this would seem to be an attractive explanation of the facts observed in these experiments there is no experimental evidence to support the hypothesis. It is not possible to present in detail all the work bearing upon the effect of freezing on living cells but interesting reviews of the question are given in papers by Maximov (21), Sacharov (27), and Stiles (25, 28), and the annual and special reports of the Low Temperature Research Station of the Food Investigation Board at Cambridge, England.

Whatever the physiological basis of this phenomenon may be, however, it is evident that certain practical uses may be made of the method as described above. The cost of the necessary equipment and of solid carbon dioxide is quite low, and with a suitable container many different strains of pathogens can be maintained for short or long periods of time. The following ways in which the method may

normal mice with 0.05 cc. of a 1:1 million dilution of infected mouse lung suspension almost invariably caused a fatal disease with typical, extensive pulmonary lesions, while with a 1:10 million dilution lesions were invariably produced with but occasional fatalities. The lethal end point of the virus is, therefore, considered to be 1:1 million, and 0.05 cc. of the 10 per cent suspension represents on the average 100,000 intranasal minimal lethal doses (M.L.D.).

Tests for virus were made by emulsifying the lungs of mice in meat infusion broth and subsequently inoculating this material into the nostrils of normal mice lightly anesthetized with ether (1, 3). Mice which received intraperitoneal or subcutaneous inoculations of virus were lightly anesthetized with ether and the material was administered from syringes through needles of 25 or 27 gauge. To avoid leakage and the consequent danger of accidental nasal contamination the site of inoculation was immediately painted with flexible collodion. The efficacy of this procedure in eliminating intranasal infection was shown by experiment in which virus was applied to the skin and the area painted with collodion. The lungs of mice so treated were tested for virus from 2 to 8 days after the application of virus to the skin. In no instance, even after serial passage, was virus demonstrable in the lungs. Other mice treated in a similar manner were tested for immunity by intranasal test 14 days later and were found to be completely susceptible.

Neutralization tests for the identification of virus were carried out with normal serum and with known immune serum according to the technique described by Francis and Magill (4).

The term, minimal lethal doses (M.L.D.), refers throughout to fatal doses as measured by intranasal infection of mice.

The Specificity of Pulmonary Lesions in Mice Following Intraperitoneal Inoculation of Virus

In a preliminary effort to determine whether the pulmonary lesions observed in mice after intraperitoneal injection of influenza virus were associated with the presence of influenza virus in the lungs, the following experiment was conducted.

To each of 4 anesthetized mice was given intraperitoneally 0.5 cc. of a 10 per cent suspension of the lungs of mice infected with the 149th mouse passage of the PR8 strain of epidemic influenza virus. On the 5th day after inoculation the mice were sacrificed and the lungs examined. Pulmonary lesions of +++, ++, ++, + severity, respectively, were noted. Passage made with suspensions of these lungs to normal mice resulted in fatal infections with complete pulmonary involvement. Neutralization tests with known immune serum demonstrated the presence of epidemic influenza virus in the involved lungs.

Attempts to induce similar changes in the lungs of etherized mice by the intraperitoneal inoculation of normal mouse lung suspensions

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were unsuccessful. On the other hand, lesions were produced in the lungs of mice after intraperitoneal injection of virus even in the absence of ether anesthesia, demonstrating that etherization played no essential rôle in the development of the lesions. Using similar procedures it was established by repeated experiments that the lesions are associated with the presence of influenza virus in the lungs of the mice although virus may be present in the absence of gross pulmonary damage.

TABLE I

Variation in Pulmonary Lesions Produced by Different, Large Intraperitoneal Doses of Virus

Group No.	Virus suspension	Quantity given	Approximate number of intranasal M.L.D.	Day of autopsy	Number of mice	Pulmonary lesions noted
	per cent	*cc.*				
1	5	0.5	500,000	6th	5	+ 0 0 0 0
2	2	0.5	200,000	6th	5	+++ ++ + 0 0
3	1	0.5	100,000	6th	5	± 0 0 0 0
4	2	0.5	200,000	6th	9	++ ++ ++ + ± 0 0 0 0
5	10	0.5	1,000,000	4th	7	± 0 0 0 0 0 0
6	10	0.1	100,000	4th	8	± 0 0 0 0 0 0 0
7	5	0.5	500,000	4th	7	++ ++ + + + + +
8	5	0.2	200,000	4th	8	++ ++ ++ ++ + + + 0
9	2	2.0	800,000	4th	5	++ ++ ++ + 0
10	2	1.0	400,000	4th	8	++ ++ ++ ++ ++ ++ + +
11	2	0.5	200,000	4th	8	+ + ± ± 0 0 0 0
12	2	0.1	40,000	4th	8	+ 0 0 0 0 0 0 0
13	1	1.0	200,000	4th	8	+ + + + 0 0 0 0
14	1	0.5	100,000	4th	8	++ + + 0 0 0 0 0
15	0.5	2.0	200,000	4th	8	+ + 0 0 0 0 0 0
16	0.5	0.5	50,000	4th	8	+ + 0 0 0 0 0 0

The Frequency of Occurrence of Virus and Lesions in the Lungs of Mice after Intraperitoneal Inoculation of Virus

In Table I is presented the incidence of pulmonary lesions in 16 groups of mice receiving relatively large doses of virus intraperitoneally. Considerable variation was observed in the frequency with which pulmonary lesions occurred in different groups of mice receiving the same dose of virus. Within the range of dosage employed in this series (50,000 to 1 million intranasal M.L.D.), no consistent relationship between the size of the intraperitoneal dose and the extent of pul-

solution of the L-dye was kept at 37°C. in a 1.4 N Na_2CO_3 solution containing R-salt for 10 days, a large proportion of the succinanilic residues had combined with R-salt, the recovered dye containing only 13.04 per cent N; a noticeable change was found after only 20 minutes. On account of this result, the authors suggested that in our experiments anaphylactic shock had been elicited not by the dye itself but by an azoprotein formed by interaction with serum protein following intravenous injection. As proof for their explanation the authors stated that they had been unable to obtain contractions of the uteri of sensitized (with dye) guinea pigs, using the dye in Schultz-Dale experiments, while the same experiment made with succinanilic acid azoprotein gave typical positive results. The failure of the dye to cause a positive Dale reaction was explained by reasoning that in this experiment there is no occasion for the formation of azoproteins in the bath (*i.e.* the bath does not contain serum proteins). Consequently no anaphylactic effect would occur, according to their concept.

In our opinion, the explanation offered by Fierz-David, Jadassohn and Zürcher for the anaphylactic shock which we induced with dyes meets with certain difficulties from the outset.[3] An obvious objection is that the splitting off of azo groups from the L-dye proceeds slowly, especially at serum pH, so that an amount of azoprotein sufficient for producing shock can hardly be formed in the few minutes elapsing between injection and anaphylactic shock, especially considering that only a small amount of the dye is injected. Another objection arises from experiments of theirs in which the L-dye was mixed with serum and the mixture kept for 24 hours at room temperature before performing the Dale test. Whereas uniformly positive results were to have been expected from the point of view of Fierz and his colleagues, actually only one out of five uteri reacted. And then, there is a convincing counter-argument in that Klopstock and Selter (8) were unable to shock sensitized guinea pigs with solutions of diazonium compounds[4] despite the high reactivity of these substances, whereas

[3] The interpretation of the authors nevertheless may well hold for the sensitization by the dye which they obtained, since in this case there would be sufficient time for the supposed formation of azoproteins.

[4] This result is understandable if one takes into account that on introduction

monary involvement was detectable. Furthermore, the variation exhibited by animals of the same group suggests that within these limits the individual reaction is perhaps of greater importance in determining pulmonary involvement than the dose of virus employed. This fact is clearly illustrated by the following experiment.

TABLE II

Titrations of Amount of Virus Present in the Lungs of Individual Mice 4 Days after Intraperitoneal Inoculation

Mouse No.	Lung lesions after intra-peritoneal inoculation	Results of intranasal inoculation of mice with different dilutions of test suspensions					
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
1	0	0 0	0 0	0 0	0 0	0 0	0 0
2	0	0 0	0 0	0 0	0 0	0 0	0 0
3	0	5 7	7 7	7 8	++ ++	++ ++	+ 0
4	0	5 7	6 7	8 9	6 9	+ +	+ 0
5	±	4 4	4 6	6 7	7 7	7 ++++	+++ +

In Tables II, III, IV, VII.

Numerals indicate day of death of test mice with typical ++++ lesions.

+ to ++++ = progressive degrees of gross pulmonary involvement in surviving mice autopsied on 10th day.

0 = no gross pulmonary lesions.

Five mice were given 0.5 cc. of 1 per cent mouse lung virus suspension (100,000 intranasal M.L.D.) intraperitoneally. The mice were sacrificed and autopsied on the 4th day after inoculation. The lung lesions were noted and titrations of the virus content of the individual lungs were made.

The results are shown in Table II. It is seen that neither virus nor lesions were present in the lungs of 2 of the mice, while virus was present in large amounts (1,000 to 100,000 M.L.D.) in the lungs of the other 3 mice although in only 1 was evidence of gross pulmonary involvement observed.

Passive sensitization experiments were made by injecting guinea pigs of approximately 300 gm. weight intraperitoneally with precipitating serum from a rabbit which had been immunized by intravenous injection of suberanilic acid azoprotein (made with horse serum) and which reacted strongly with an antigen prepared from *p*-aminosuberanilic acid and chicken serum. Three guinea pigs sensitized with 1 cc. of the immune serum were injected intravenously the next day with 0.33 mg. of the suberanilic acid dye (made according to the method of Fierz) in 1 cc. Of these animals one died in acute

TABLE I

Animals Tested by Intravenous Injection of Solutions of Resorcinoldisazo-p-Succinanilic Acid Prepared According to the Method of Fierz-David and Coworkers

Animals sensitized with azoprotein made from *p*-aminosuccinanilic acid				Normal animals			
Guinea pig No.	Amount of dye injected	Subsequent change in body temperature	Result, symptoms	Guinea pig No.	Amount of dye injected	Subsequent change in body temperature	Result, symptoms
	mg.	°C.			mg.	°C.	
1	2	−1.0	Medium to severe	10	2	+0.3	Negative
2	1		†8 min.	11	2	−0.7	"
3	1		†5 "	12	2	−0.8	"
4	1		†7 "				
5	1	−1.0	Slight				
6	1	−1.8	Medium				
7	1		†23 min.				
8	1	−0.8	Slight				
9	0.33	−2.9	Slight to medium				

† means death of animal.

shock in 3 minutes, one had severe and one medium anaphylactic symptoms. Three animals injected with 2 cc. of the immune serum died in 3 to 5 minutes on intravenous injection of 1 mg. of the dye the next day.

Likewise three out of four guinea pigs passively sensitized with 2 cc. of rabbit immune serum to succinanilic acid azoprotein died of anaphylactic shock within 4 minutes when they were reinjected intravenously the next day with 2 mg. of F-succinanilic acid dye.

Injection of either dye into non-sensitized animals caused no symptoms.

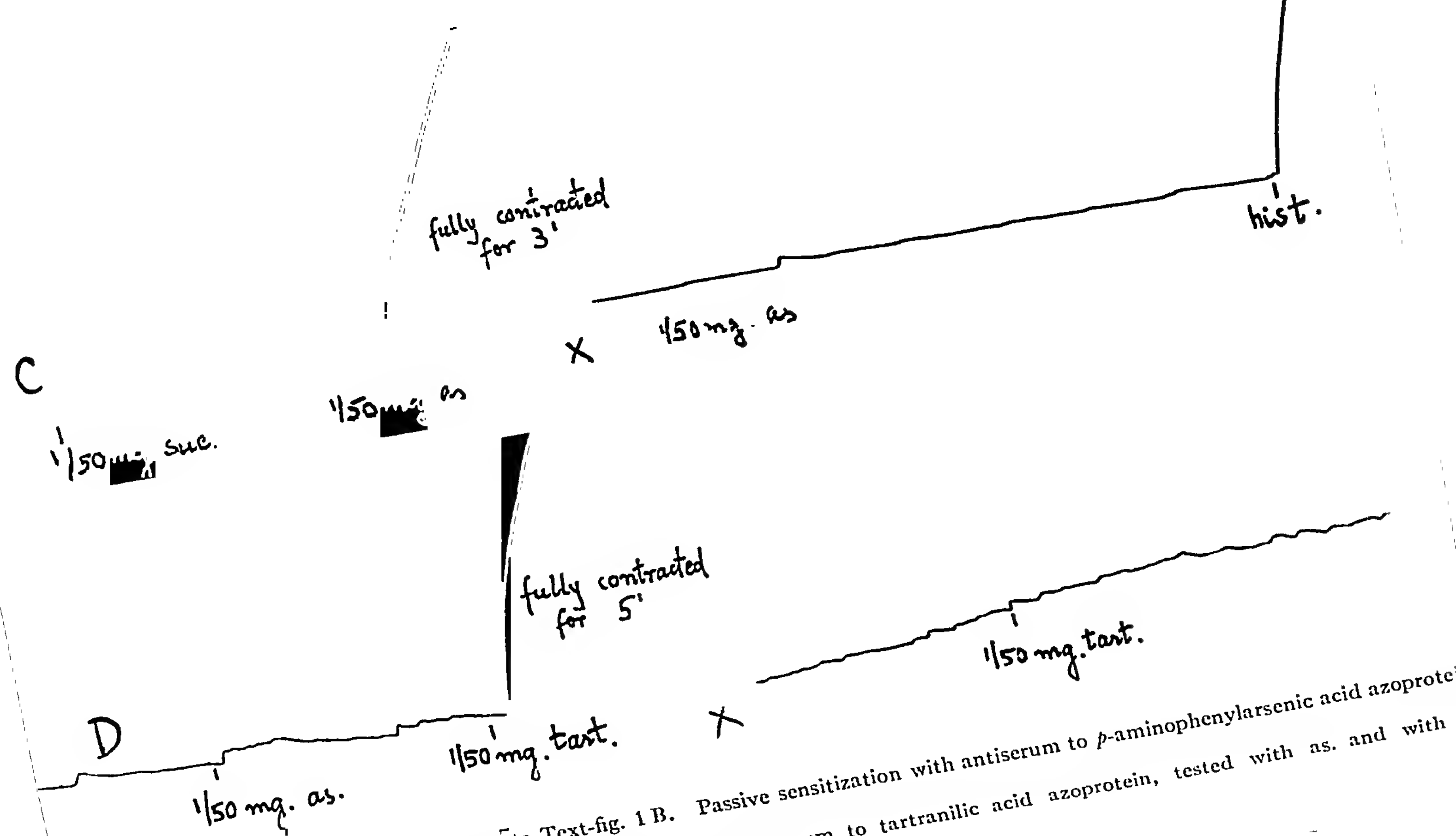
Owing to variation in individual mice the minimal intraperitoneal dose of virus which results in the appearance of lesions or virus in the lungs has not been subject to determination with the same accuracy as in the case of virus administered intranasally. There has been, nevertheless, a relatively sharp boundary between effective and non-effective doses. The injection of 100,000 M.L.D. intraperitoneally is invariably followed by the appearance of virus in the lungs of a large proportion of the inoculated mice. With 10,000 M.L.D. virus was recovered in only one of several attempts. When intraperitoneal doses of 1,000 M.L.D. were given, virus was not demonstrable in the lungs over a period of 1 to 4 days, even with the secondary passage technique employed by the Russian investigators (2). Paralleling to a certain extent the presence of virus in the lungs, lesions have been observed in the lungs, but for one exceptional instance, only when doses greater than 10,000 M.L.D. have been given intraperitoneally.

It is readily apparent, therefore, that the intraperitoneal injection of influenza virus is followed by the appearance of virus and the associated lesions in the lungs of mice only after large amounts of virus are used. When less than 10,000 intranasal M.L.D. were given intraperitoneally, no evidence of the presence of virus in the lungs was obtained.

Search for Virus and Lesions in the Lungs of Mice at Daily Intervals after Intraperitoneal Inoculation

The foregoing results were based on observations made to a great extent on the 4th day after intraperitoneal inoculation of virus. To determine the length of time through which virus and lesions could be detected in the lungs after the introduction of virus into the peritoneal cavity, the following experiments were conducted.

Each of 15 mice in series A was given 0.5 cc. of 2 per cent virus suspension (200,000 M.L.D.) intraperitoneally with the aid of ether anesthesia. To 29 mice of series B, 0.5 cc. amounts of 10 per cent virus suspension (1 million M.L.D.) were given intraperitoneally without the use of ether. Groups of 3 to 5 mice were killed at daily intervals, the presence of pulmonary lesions noted, and the pooled lungs of each group made into 20 per cent suspensions which were then tested for the presence of virus by intranasal inoculation into 3 normal mice. These test mice were observed for 10 days and the day of death and the lung lesions were recorded. All survivors were sacrificed and examined on the 10th day. The results are tabulated in Table III.



TEXT-FIG. 2 C. Specificity test, converse to Text-fig. 1 B. Passive sensitization with antiserum to p-aminophenylarsenic acid azoprote reaction to as. but not to suc.

TEXT-FIG. 2 D. Specificity test. Passive sensitization with antiserum to tartranilic acid azoprotein, tested with as. and with resorcinoldisazo-p-tartranilic acid.

In both series of mice virus was recovered from the lungs as early as 24 hours after its intraperitoneal administration and, as judged by the time of death of the test mice, reached its height on the 4th day, at which time pulmonary lesions were first observed. In series B a gradual decrease in the amount of virus occurred after the 4th day so that by the 7th day virus was not detectable, and the lesions observed

TABLE III

Search for Virus and Pulmonary Lesions in the Lungs of Mice at Daily Intervals after Intraperitoneal Inoculation of Virus

Day mice killed after intraperitoneal inoculation	Series A					Series B						
	Number of mice in group	Lesions noted			Day of death of test mice with ++++ lesions	Number of mice in group	Lesions noted					Day of death of test mice with ++++ lesions
1st	3	0	0	0	6, 7, 8	3	0	0	0			8, 8, 8
2nd	3	0	0	0	4, 4, 5	3	0	0	0			6, 6, 7
3rd	3	0	0	0	3, 6, 6	4	0	0	0	0		6, 6, 7
4th	3	+	+	0	3, 3, 4	5	+	+	+	+	0	5, 5, 6
5th	3	++	++	0	4, 4, 5	5	++	0	0	0	0	7, 8, 8
6th	—		—		—	5	+	+	0	0	0	8, 9, 10
7th	—		—		—	4	++	+	+	0*		0, 0, 0

* Lesions of a greyish appearance commonly seen in mice surviving sublethal intranasal doses.

in the lungs at that time were of a pale greyish appearance characteristic of the healing process. It was also of interest that the use of ether exerted no apparent influence upon the outcome of the experiment.

Subsequent studies were then conducted to ascertain by titration the relative amounts of virus present in the lungs of mice at daily intervals after intraperitoneal injection.

Intraperitoneal injections of 0.5 cc. amounts of 1 per cent virus suspension were made in 21 mice. The animals were sacrificed in groups of 3 at daily intervals from 1 to 7 days after inoculation. No pulmonary lesions were observed. The lungs of each group were pooled and broth suspensions in dilutions of from 10^{-1} to 10^{-6} were inoculated intranasally into test mice. The mice were observed for 10 days at the end of which time the survivors were sacrificed and the presence or

of normal guinea pigs none of the dyes caused an effect with quantities even of 0.5 mg. in the 20 cc. bath.

Similar reactions were secured in passive sensitization with a rabbit immune serum for suberanilic acid azoprotein, the tests being made with the corresponding dye.

These striking results suggested trying analogous experiments with some other azodyes for which immune sera prepared with the corresponding azoproteins were available. Positive effects were found with azodyes made according to the method of Fierz from resorcinol and *p*-aminophenylarsenic, *p*-aminotartranilic acids and *m*-aminobenzoyl glycine, while the dyes made from *m*-aminobenzenesulfonic and *m*-aminobenzoic acids were ineffective. Also here the specificity of the reactions could be demonstrated unambiguously (Text-fig. 2 D). It was further observed that the positive cases were those in which addition of Ca salts to a 0.01 per cent solution of the dye caused precipitation within a few hours at the most, whereas in the negative cases precipitation appeared very slowly (visible on the next day), or not at all. Since the bath in which the uteri are suspended contains calcium, one may surmise that this property of the dyes may be of influence in obtaining positive results;[6] however the number of instances investigated is relatively small. With the concentrations obtaining in the tests, no precipitation was observed in the bath during the experiments; only with the suberanilic acid compound, if the concentration was high enough, a turbidity was soon seen and later a precipitate appeared.

SUMMARY

From the experiments presented, it follows that the specific precipitation and the production of anaphylactic shock with certain azodyes, as described previously, is due to these substances themselves and is not dependent upon formation of azoproteins by interaction of the dyes with proteins in the test tube or the animal body.

Besides these, some other azodyes which in our tests did not give precipitation with corresponding immune sera were also found, in very small quantities, to induce anaphylactic contraction of the uterus of sensitized guinea pigs.

[6] On the possible relation of the precipitability of dyes by means of metal salts to the degree of dispersion, *vide* (12).

absence of pulmonary lesions noted. The results of the titrations are presented in Table IV.

It is seen that while virus was present in the lungs 24 hours after its intraperitoneal administration the amount was comparatively small.

TABLE IV

Titration of Amounts of Virus Present at Daily Intervals in the Lungs of Mice after Intraperitoneal Inoculation

Day after intraperitoneal inoculation	Day of death of test mice with ++++ lesions in different dilutions					
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
1st	9	0	0	0	0	0
	++	0	0	0	0	0
	+	0	0	0	0	0
2nd	4	4	4	5	8	++
	4	4	4	8	++++	++
	4	8	6	8	++	+
3rd	4	4	5	7	7	++
	4	4	5	8	7	0
	5	6	5	9	9	0
4th	7	7	10	++	0	0
	7	8	++++	+	0	0
	8	8	++++	0	0	0
5th	5	5	9	+++	+	+
	7	6	++++	++	+	0
	8	7	++++	++	0	0
6th	4	5	5	7	+++	+
	4	5	6	10	++	0
	5	6	10	++++	++	0
7th	7	8	+++	+	0	0
	8	10	++	+	0	0
	8	10	+	0	0	0

At 48 and 72 hours, however, as much as 100,000 M.L.D. of virus was detected in the lungs. Thereafter, virus was found in the lungs throughout the course of the experiment although in somewhat smaller amounts.

Since in the preceding experiment no end point was reached as to the number of days virus persisted in the lungs, the procedure was repeated and extended to 11 days. The results were quite parallel to those given above, especially in regard to the low titer of virus in the lungs at the end of 24 hours and the rapid increase to a maximum in 48 to 72 hours. In addition, sufficient virus was present in the lungs of mice 8, 9, and 10 days after intraperitoneal injection for a dilution of 1:1,000 to cause fatal infection in test mice, while on the 11th day no virus was demonstrable. Obviously, however, the duration of virus in the lungs of mice in different experiments is variable.

The presence of small amounts of virus in the lungs on the 1st day after intraperitoneal inoculation, the rapid increase in amount on the 2nd and 3rd days, and the persistence of virus over a prolonged period suggest that multiplication of the virus has taken place in the lungs. Owing to the large amounts of virus injected, the possibility remains, however, that the virus has selectively localized in the lungs. This hypothesis would demand acceptance of the idea that either the virus is capable of surviving in the body in a relatively inert state for several days or that it is multiplying in other parts of the body and being transported to the lung. In any case, it is apparent that following intraperitoneal inoculation of influenza virus the lung of a mouse may, without showing any evidence of pulmonary injury due to virus, contain virus in sufficient amount to kill 100,000 mice if inoculated intranasally.

Observations upon the Distribution of Virus after Intraperitoneal Injection

In order to evaluate the significance of the recovery of relatively large amounts of virus from the lungs of mice receiving influenza virus intraperitoneally, it was necessary to study further the distribution of virus in other organs and in the circulating blood. If virus is consistently present in the blood, its presence in other organs and the mode of its transport to the lungs is readily explained.

Presence of Virus in the Blood.—

Three groups of 5 mice each were given approximately 100,000 M.L.D. of virus intraperitoneally. At 1, 3, and 4½ hour intervals, respectively, 10 drops of blood

bile by mouth or in a dog with complete biliary obstruction. The osteoporosis develops because of lack of absorption of vitamin D and this state is corrected or prevented by the presence of bile salts in the intestine (1).

The osteoporosis seems to be due to faulty absorption. The abnormal *bleeding* of a bile fistula is not a simple matter of absorption as this condition does not develop in biliary obstruction. Substances formed in the liver, excreted in the bile and reabsorbed, appear to be necessary for normal prothrombin formation.

Likewise this *inadequate hemoglobin production* due to the presence of a bile fistula is not a simple matter of intestinal absorption related to the bile in the intestine. After the bile fistula is produced we may feed bile or not but this does not appreciably modify the hemoglobin production. One notes in the tables below that the output of hemoglobin is the same whether during bread periods the food is mixed with dog bile or given for a 10 week period with no bile at any time (dog 29-66, Table 3–continued). Iron by mouth given during periods with or without bile shows the same response.

One may choose to believe that once the *cycle* of bile salt secretion, intestinal absorption and resecretion by the liver is interrupted, certain abnormalities develop some of which are not corrected by bile feeding. This cycle of the bile salts may involve other fractions in the bile and the interruption of this cycle of secretion and reabsorption may disturb both intestinal mucosa and hepatic epithelium.

In the normal dog there is a more or less *continuous circulation* of bile salts and we have always felt that the *internal* part of this cycle was as important as the external (intestinal) portion. We may argue that the normal state of the liver cell is in part dependent upon this continuous bile salt cycle, and when this circulation is interrupted for a considerable part of each day, the liver epithelium suffers and is unable to assemble as skillfully as usual all the building stones which go eventually to form the complex hemoglobin molecule. There is no reason to suspect the *bone marrow* which is normal histologically.

Methods

The general methods used in the anemia experiments have been carefully described and various method controls are given in detail (10). The bile fistula

were obtained from the tail vein of each mouse of one group. Blood from each group was pooled, ground with alundum in 1.0 cc. of broth, and the supernatant fluid was inoculated into the nostrils of 3 normal mice. When the mice which received the blood were killed 3 days later no lung lesions were seen, but a second serial passage made with lungs of these mice resulted in death of all second passage mice with influenza virus infection as confirmed by neutralization tests.

Thus the presence of virus in the blood was demonstrated from 1 to 4½ hours after its introduction into the peritoneal cavity. Using the same procedures, however, repeated attempts to demonstrate virus in the blood from 24 hours to 6 days after inoculation have been uniformly unsuccessful.

TABLE V

Virus Demonstrated in Blood and Lungs of Individual Mice after Intraperitoneal Inoculation

Virus in blood 3 hrs. after inoculation..	+N	0	0	0	0	0	+	+	0	+
Virus in lungs 3 days after inoculation.	+	0	0	+	0	+N	0	+	+N	0

In Table V.
+ = virus present.
0 = no virus present.
N = positive neutralization test with influenza immune serum.

A further experiment was done in which 9 mice were injected intraperitoneally with approximately 10,000 intranasal M.L.D. by the same technique as above. The blood of each mouse was tested for the presence of virus 3 hours after inoculation and the lungs of each mouse were tested on the 3rd day after injection. Virus was detected in the blood of 4 of the 9 mice and the nature of the virus confirmed by neutralization tests in mice. No virus was found in the lungs. This dose of virus is, of course, below the threshold which ordinarily results in demonstrable virus in the lungs.

The results of a similar experiment in which 100,000 M.L.D. of virus were given intraperitoneally to 10 mice are briefly summarized in Table V. Virus was demonstrated in the blood of 4 of the 10 mice 3 hours after intraperitoneal injection, and in the lungs of 5 of the 10 mice 3 days later. In 2 of the mice virus was found in the blood and again subsequently in the lungs. In 2 of the mice virus was found

TABLE 1

Hemoglobin Production in Anemia Depressed by Bile Fistula

Dog 32-1. Coach, male, adult.

Diet, daily average intake	Bile given daily	Experimental period	Food consumed av.	Weight av.	Blood Hb. level av.	Hb. removed per wk. av.	Total net Hb. output 2 wks.
gm.	*cc.*	*wks.*	*per cent*	*kg.*	*per cent*	*gm.*	*gm.*
Bread 260, salmon 117	0	6	99	13.0	46	10.4	
Apricots 100, br. 225, salm. 125	0	2	100	12.1	52	27.8	34.2
Bread 263, salmon 125	0	2	100	12.3	47	10.1	
Fe 40 mg., bread 300, salmon 100	0	2	100	12.4	59	42.3	68.7
Bread 325, salmon 100	0	2	100	12.5	46	12.9	
Pig liver 300, bread 300	0	2	100	13.1	59	51.3	97.0
Bread 375, salmon 92	0	3	100	13.5	47	15.5	
Renal Bile Fistula Operation							
Bread 375, salmon 125	0	4	92	13.4	44	4.1	
Pig liver 300, bread 300	40	2	80	12.3	48	22.2	26.2
Bread 288, salmon 200	40	2	74	12.1	44	0	
Fe 40 mg., bread 225, salmon 200	70	2	100	12.0	50	15.7	21.6
Bread 225, salmon 175	70	2	91	11.9	46	3.2	
Bread 336, salmon 139	100	9	95	13.0	48	8.5	
Fe 40 mg., bread 350, salmon 150	100	2	90	13.2	54	17.4	18.1
Bread 350, salmon 150	100	2	95	12.9	54	8.7	
Apricots 100, br. 300, salm. 125	100	2	100	13.5	50	16.4	18.7
Bread 350, salmon 100	100	2	89	13.3	45	10.0	
Fe 400 mg., bread 300, salmon 200	50	2	78	12.8	66	46.0	59.1
Bread 225, salmon 200	91	3	97	12.6	51	6.0	
Pig liver 200, bread 263	80	2	77	11.2	57	26.1	51.6
Bread 225, salmon 200	80	3	88	10.7	41	6.8	
Bread 225, salmon 200	80	6	92	11.0	41	4.2	
Fe 40 mg., bread 300, salmon 175	115	2	100	11.7	50	16.5	27.7
Bread 300, salmon 150	125	2	96	12.0	43	5.8	

in the blood but was not in the lungs 3 days later. In 3 instances virus was present in the lungs although not demonstrable in the earlier tests of the blood. In 3 instances virus was not demonstrated in either the blood or lungs. These experiments demonstrate that shortly after the intraperitoneal inoculation of virus, the agent can be recovered from the circulating blood. Little information is elicited, however, as to the relation between circulating virus and the presence of virus in the lungs. That virus can be obtained from the lung following intravenous injection will be subsequently shown (Table VIII). The significance of the results will be discussed more thoroughly in that connection.

Presence of Virus in Organs Other than the Lung.—In an experiment to determine the distribution of influenza virus in the body after the intraperitoneal injection of large doses of the agent, mice so treated were killed after 3 hours, and others on the 3 following days. In addition to the blood and peritoneal washings, the lungs, nasal turbinates, liver, spleen, and kidneys were examined for the presence of virus, utilizing 2 serial passages in normal mice for this purpose.

Blood was examined by the procedure previously described. Peritoneal washings were obtained by flushing the abdominal contents with approximately 1.0 cc. of broth which was then inoculated in 0.05 cc. amounts intranasally to test mice. The abdominal organs were vigorously washed 5 times in 20 cc. amounts of Locke's solution, an amount thought to be sufficient to eliminate any virus that might be present on the surface of the organs. A 10 per cent suspension of each organ in broth was then given intranasally to mice.

Three hours after intraperitoneal inoculation, virus was present in small but demonstrable amounts in the blood; it was present in larger amounts in the peritoneal washings and lungs, but not demonstrable in the nasal turbinates. On all 3 days thereafter virus was recovered from the peritoneal washings and lungs but not from the turbinates.

Although positive results were obtained in examination of liver, spleen, and kidneys the difficulty in eliminating direct contamination from the peritoneal cavity makes their evaluation uncertain. This is emphasized by a control experiment in which 2 mice were killed and immediately given 1.0 cc. of 1 per cent virus suspension intraperitoneally. After abdominal massage, liver, spleen, and kidney were removed, subjected to the same amount of washing, and then tested for the presence of virus. Virus was recovered from all the organs.

common duct is occluded and the gall bladder is not distended but opens freely into the renal pelvis. There are no calculi. The renal pelvis shows many mononuclears in the stroma just below the epithelial covering which is normal and intact. *Kidneys* are normal but for the scars related to the fistula tract. Histological sections show normal structures. Gastro-intestinal tract is normal except for brown pigmentation of the muscle coats of the small intestine. Histological sections show a conspicuous deposit of fine grains of yellow pigment (6) within the smooth muscle cells. Pancreas normal in all respects but for a deposit of similar pigment in phagocytes and acinar epithelium.

Bones show no osteomalacia and a normal structure. Fat marrow is abundant in ulnae, radii and tibiae. The humeri and femora show a brick red cellular hyperplastic marrow as do the ribs and vertebrae. Histological sections show normal marrow cells and the usual picture of marrow hyperplasia in long continued anemia (7).

Iron analyses of various tissues show the expected low values found in long continued anemia (2), liver 1.5 mg. per cent, spleen 4 mg. per cent, kidney 2.5 mg. per cent, ribs 2 mg. per cent, vertebrae 4.5 mg. per cent.

Dog 32-2 (Table 2) is a very satisfactory animal—a litter mate of dog 32-1 above. This dog remained active and healthy for 3 years after the fistula operation. A mixture of dog and ox bile was given daily in liberal amounts. This dog since the fistula operation produced about one-half as much hemoglobin on standard diets as during the normal control fore period. It is to be noted especially that food consumption at all times is 100 per cent and the loss of weight during the greater part of this period is less than 1 kilo. All these factors make this almost a perfect experiment and the values recorded deserve especial emphasis.

Colloidal iron given intravenously (Table 2–continued) shows a return of 86 gm. hemoglobin compared with the theoretical return of 88 gm. assuming that all the injected iron reappeared in the new formed hemoglobin. In other words this dog uses iron by vein just like a normal dog but produces much less than normal when the iron is fed. This is strong evidence that the absorption of iron is seriously disturbed. A similar experiment is recorded in Table 3–continued.

The last liver feeding experiment (Table 2–continued) shows a large intake of bile spread over 12 hours in an attempt to show whether this would modify the output of new hemoglobin. In this

The tendency of the virus to remain in the peritoneal washings and in the abdominal viscera suggested that the virus is taken up by the serosal cells of the peritoneum and the capsules of the abdominal organs. This suggestion was tested in the following experiment.

Six mice were injected intraperitoneally with 100,000 M.L.D. of virus. The mice were bled and killed in groups of 2 on the 1st, 3rd, and 6th days following inoculation. Peritoneal washings were obtained in the manner already described. The intestines were removed and abdominal cavities vigorously flushed 14 times with 12 cc. of saline per flushing, after which pieces of mesentery and parietal peritoneum were made into 10 per cent suspension for inoculation into test mice. Peritoneal washings and suspensions of blood, lungs, and turbinates were likewise inoculated. Except in cases where death had already occurred or the test mice were obviously ill, on the 4th day following inoculation 2 of the group were killed (A) and secondary passage made to 2 additional mice (C); the remaining 2 of the 4 original test mice (B) were kept until the 10th day when survivors were killed and lesions observed.

TABLE VI

The Distribution of Virus after Intraperitoneal Injection in Mice

Organs examined	Deaths and lesions in test mice								
	1st day			3rd day			6th day		
	A	B	C	A	B	C	A	B	C
Blood	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0
Peritoneal washings	6, 6	8, 8	– –	+ +	10, 10	4, 4	0 0	0 0	0 0
Mesentery and parietal peritoneum	+ 0	++ 0	4, 6	4, 4	4, 4	– –	+ +	7, 7	5, 5
Lungs	5, 5	6, 7	– –	4, 4	4, 5	– –	0 0	0 0	0 0
Turbinates	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0

In Tables VI and VIII.

A = day of death with ++++ lesions or lesions seen when mice were killed on 4th day after inoculation; primary passage mice.

B = day of death with ++++ lesions or lesions seen when mice were killed on 10th day after inoculation; primary passage mice.

C = day of death with ++++ lesions or lesions seen when mice were killed on 10th day after inoculation; secondary passage mice.

As observed in Table VI virus was found in the peritoneal washings as long as 3 days after its injection, while in the cells of the mesentery and parietal peritoneum virus was demonstrable as late as 6 days after

TABLE 2

Hemoglobin Production in Anemia Depressed by Bile Fistula

Dog. 32-2. Coach, female, adult.

Diet, daily average intake	Bile given daily	Experimental period	Food consumed av.	Weight av.	Blood Hb. level av.	Hb. removed per wk. av.	Total net Hb. output 2 wks.
gm.	*cc.*	*wks.*	*per cent*	*kg.*	*per cent*	*gm.*	*gm.*
Bread 320, salmon 96	0	6	100	14.1	48	2.0	
Apricots 75, br. 200, salm. 100	0	2	100	13.7	53	28.8	57.6
Bread 263, salmon 100	0	2	100	14.0	43	4.0	
Fe 40 mg., bread 275, salmon 100	0	2	100	14.0	57	36.6	71.1
Bread 300, salmon 100	0	2	96	13.8	52	3.0	
Pig liver 300, bread 350	0	2	100	14.0	60	44.5	102.0
Bread 367, salmon 100	0	3	100	14.2	46	7.7	
Lextron, bread 375, salmon 75	0	2	100	14.1	63	51.1	129.1
Bread 400, salmon 75	0	2	100	14.2	49	17.5	
Renal Bile Fistula Operation							
Bread 450, salmon 75	70	4	100	14.5	44	4.2	
Fe 40 mg., bread 450, salmon 75	70	2	100	14.4	56	23.0	53.9
Bread 450, salmon 75	65	2	100	14.2	44	12.2	
Pig liver 300, bread 300	70	2	100	13.8	50	29.7	57.2
Bread 375, salmon 75	70	3	100	14.0	47	6.1	
Bread 400, salmon 75	70	14	100	13.6	47	10.0	
Lextron, bread 400, salmon 75	70	2	100	13.4	56	28.2	39.0
Bread 400, salmon 75	70	3	100	13.4	51	13.6	
Apricots 100, br. 337, salm. 75	70	2	100	13.3	55	20.7	20.3
Bread 400, salmon 75	70	2	100	13.0	46	9.5	
Fe 400 mg., bread 400, salmon 75	100	2	100	13.1	53	31.9	47.7
Bread 400, salmon 75	70	3	100	12.9	50	17.0	
Bread 400, salmon 75	70	3	100	12.4	43	7.4	
Pig liver 300, bread 350	70	2	100	12.5	51	26.1	33.5
Bread 400, salmon 75	70	3	100	12.4	43	7.4	
Bread 450, salmon 100	100	5	100	12.2	49	8.6	
Fe 21 mg.,* bread 400, salmon 75	70	2	100	12.3	54	37.4	86.0
Bread 400, salmon 75	70	3	100	12.0	54	18.1	

* Iron given intravenously—86 gm. Hb. is 97 per cent of theoretical return.

injection. The latter observation shows clearly the capacity of the virus to survive and perhaps to multiply in the peritoneal cells. In contrast to these results is the absence of virus from the blood and nasal turbinates. Virus was not recovered from the lungs after the 3rd day, a result perhaps due to the factor of individual variation in the mice.

The Development of Resistance in Mice Receiving Large Doses of Influenza Virus Intraperitoneally

It has been clearly shown that as a result of the intraperitoneal inoculation of mice with the larger doses of influenza virus a high concentration of the virus and gross pathological changes are found in the lungs of the animals. In view of these facts it is noteworthy that in a large series of observations upon mice so inoculated no deaths from influenza virus infection have occurred. This result is even more striking when it is recalled that as much as 100,000 intranasal lethal doses of virus may be recovered from the lung of a mouse which appears perfectly healthy after the intraperitoneal injection of virus. It must be assumed either that the virus reaches the lungs by a route which prevents its attacking the pulmonary tissues in the same manner as after intranasal inoculation (2), or that an alteration in the reactivity of the animal host occurs as a result of the pararespiratory injection so as to inhibit the virus from exerting its full pathogenic influence upon the pulmonary tissues to which it has gained access. The fact that the pulmonary lesions observed after intraperitoneal inoculation of virus are rarely of more than moderate extent seems to support the latter point of view. Moreover, the animals become solidly immune to subsequent intranasal tests with as much as 100,000 M.L.D. of virus.

The following experiment was undertaken to determine whether after the intraperitoneal inoculation of virus any evidence could be obtained of a rapidly developing resistance which might serve to protect mice against the characteristic virus effect.

Suspensions of 2 per cent mouse lung virus were prepared and stored in a freezing mixture (5) at −79° C. until used. Every day for 5 days a different group of 10 mice each was given 0.5 cc. of the virus suspension intraperitoneally. On the 3rd day all mice, regardless of whether or not they had yet received the virus

TABLE 3

Hemoglobin Production in Anemia Depressed by Bile Fistula

Dog 29-66. Bull terrier, male, adult.

Diet, daily average intake	Bile given daily	Experimental period	Food consumed av.	Weight av.	Blood Hb. level av.	Hb. removed per wk. av.	Total net Hb. output 2 wks.
gm.	*cc.*	*wks.*	*per cent*	*kg.*	*per cent*	*gm.*	*gm.*
Bread 400, salmon 75	0	6	100	15.3	49	2.0	
Fe 40 mg., bread 400, salmon 75	0	2	100	14.9	54	26.3	52.0
Bread 400, salmon 75	0	2	100	15.0	47	3.8	
Pig liver 300, bread 350	0	2	100	15.6	58	45.5	104.7
Bread 400, salmon 75	0	3	100	15.7	46	7.2	
Apricots 100, bread 350, salmon 75	0	2	100	16.0	50	17.0	46.7
Bread 400, salmon 75	0	2	96	16.2	51	10.3	
Bread 400, salmon 82	0	10	99	16.2	46	11.0	
Bread 383, salmon 125	0	6	99	16.6	49	8.6	
Fe 40 mg., bread 375, salmon 125	0	2	100	16.3	55	28.9	38.0
Bread 375, salmon 125	0	2	100	16.2	41	7.3	
Pig liver 300, bread 275	0	2	95	15.7	65	45.6	70.2
Bread 400, salmon 125	0	3	100	15.6	51	7.4	
Dog liver 300, bread 280	0	2	100	15.4	61	46.0	99.0
Bread 400, salmon 100	0	3	100	15.3	49	17.0	
Renal Bile Fistula Operation							
Bread 425, salmon 115	50	11	99	14.3	47	7.0	
Apricots 100, bread 350, salmon 150	50	2	100	14.6	51	15.1	28.6
Bread 400, salmon 75	50	2	100	14.5	53	13.1	
Fe 40 mg., bread 400, salmon 75	50	2	100	14.7	49	19.6	21.7
Bread 400, salmon 75	50	2	100	14.2	45	5.2	
Pig liver 300, bread 350	50	2	100	13.7	56	23.8	34.4
Bread 450, salmon 75	50	3	96	14.4	45	7.2	
Pig kidney 300, bread 375	40	2	100	13.8	44	11.4	30.1
Bread 400, salmon 130	45	3	97	13.9	48	14.1	
Pig spleen 300, bread 300	40	2	100	13.1	59	25.0	20.6
Bread 375, salmon 125	40	2	100	13.0	50	0	

intraperitoneally, were given 0.05 cc. amounts of a 1:100,000 dilution of virus intranasally. In addition one group of 10 mice received only the intranasal inoculation, while another group received only the intraperitoneal injection of virus. The mice were observed for 10 days at which time all survivors were sacrificed and the lungs examined. The results are summarized in Table VII.

As evidenced by the fact that all survived and that only low grade pulmonary lesions were found at autopsy, the mice which received the intraperitoneal inoculation of virus 2 days before intranasal infection exhibited a well marked resistance. This is especially im-

TABLE VII

The Effect of Intraperitoneal Injection of a Large Dose of Virus before and after the Intranasal Inoculation of a Small Infective Dose of the Same Virus

Time relation of intraperitoneal to intranasal inoculation	Day of death with ++++ lesions or lesions seen in mice surviving 10 days after intranasal inoculation									
2 days before	++	++	+	+	+	+	+	±	0	0
1 day before	7,	7	++	++	+	+	+	+	+	+
Same day	6,	6,	7	+++	+++	++	++	++	++	++
1 day after	7,	7,	7,	7,	7,	7,	7	++	++	0
2 days after	6,	7,	7,	7,	7,	7,	9,	9,	10	++++
Intranasal only	7,	7,	7,	8,	8,	8	+++	++	++	++
Intraperitoneal only	+	+	+	+	0	0	0	0	0	0

pressive when one considers that the intranasal infection was given at about the time when the amount of virus recoverable from the lung following intraperitoneal injection reaches its maximum. Although to a lesser extent, a similar effect was observed in the mice receiving virus intraperitoneally 24 hours before intranasal infection. Thereafter, the severity of the pulmonary lesions and the frequency of death increased so that animals receiving intraperitoneal virus after the intranasal infection showed no evidence of resistance. Similar results were obtained when these observations were repeated using 10 per cent virus concentration for the intraperitoneal injection.

Since the resistance developed so rapidly it was of further interest to ascertain what correlation, if any, obtained between the active resistance of the animals and the development of circulating antiviral substances. Accordingly, each of 45 mice was given the equivalent

form hemoglobin in anemia after the fistula operation in comparison to pre-operative periods (Table 6).

This dog shows interesting figures during long periods of bread feeding with and without bile. Table 3-continued during a bread feeding period of 10 weeks *without any bile* shows an average weekly output of 12.4 gm. hemoglobin. The period of 11 weeks immediately following with the same bread diet and food intake *plus* 100 cc. bile and 2 gm. of sodium taurocholate daily indicates an average weekly output of 14.2 gm. hemoglobin. Some months later (Table 3-continued) during a 10 weeks' diet period of the same character *plus* 150 cc. of bile, the dog shows an average weekly output of 9.7 gm. hemoglobin. During another 11 week period shortly after the bile fistula operation (Table 3) with a similar diet *plus* 50 cc. bile, the dog shows an average weekly output of 7.0 gm. hemoglobin. It is obvious therefore that the actual presence of bile in the food given daily does not appreciably modify the hemoglobin production of a dog on the standard bread and salmon diet.

When bile in large amounts is given by mouth during the day time (9 a.m., 12 m., 4 and 8 p.m.) during standard liver feeding experiments (Table 3-continued) we note some increase in this dog (77 gm. hemoglobin total net output) as compared with many earlier feeding experiments (48.5—39.2—41.0—34.4 gm. hemoglobin). In another experiment (Table 2) another dog shows little if any increase in hemoglobin production under similar conditions of bile feeding.

Iron given by *mouth* (Table 3-continued) whether with or without bile shows the same output of new hemoglobin per week—58.2 and 57.0 gm., respectively. These figures are approximately two-thirds the normal average return of 90 gm. hemoglobin in the anemic control dog (see Table 6).

Iron given by *vein* (Table 3-continued) gives 80 and 98 per cent of the theoretical return if all the injected Fe is returned quantitatively within new hemoglobin.

Clinical History.—Table 3. Dog 29-66. Adult male bull terrier. Born Sept. 7, 1928. Experimental anemia from Sept., 1930, to Feb., 1932. Routine anemia experiments (Table 3). Dog returned to normal blood hemoglobin level for bile fistula operation.

Apr. 18, 1933. Renal bile fistula operation. Uneventful recovery.

of 50,000 to 100,000 intranasal M.L.D. intraperitoneally. At intervals of approximately every 3 days from 1 to 27 days thereafter, groups of 5 mice were bled from the tail veins. The serum of each group was pooled and tested for the presence of antibodies to influenza virus by means of the protection test in mice. No antibodies were detected in the serum taken 1 or 3 days after intraperitoneal inoculation. On the 5th, 7th, and 10th days sufficient antibody was present in undiluted serum to protect test mice against fatal infection with 1,000 lethal doses of virus even though some pulmonary involvement was observed. On the 13th day the serum had a protective titer between 1:100 and 1:200, and this level was maintained until the termination of the experiment on the 27th day.

It appears, therefore, that the resistance exhibited to intranasal virus infection by mice which receive large doses of influenza virus intraperitoneally is effective at the very time maximal amounts of virus tend to collect in the lungs and, on the basis of the preceding experiment, before neutralizing antibodies are detectable in the blood. This apparently non-specific resistance may possibly represent an example of the interference phenomenon similar to that described for yellow fever virus (6), when the neurotropic strain of virus given to monkeys intraperitoneally protects against an otherwise fatal infection with the viscerotropic strain of virus. Further investigation of the nature of the resistance observed in the present experiments will be required.

Distribution of Virus after Subcutaneous or Intravenous Administration

The foregoing experiments have revealed that after the intraperitoneal inoculation of mice with large amounts of influenza virus the virus can be recovered from the blood for a short time and from the lungs in relatively high concentrations for a number of days. When the same procedures were applied after the subcutaneous inoculation of similar amounts of virus, virus was not demonstrable in the blood or lungs of the mice. Moreover, one large dose of virus subcutaneously results not only in a less effective active immunity among the treated mice but also in a lower titer of neutralizing antibodies in the blood than when the same amount of virus is given by the intraperitoneal route. Virus was readily recovered, however, from the re-

TABLE 4

Hemoglobin Production Much below Normal Due to a Bile Fistula

›g 26-19. Bull terrier, male, adult.

Diet, daily average intake	Bile given daily	Experimental period	Food consumed av.	Weight av.	Blood Hb. level av.	Hb. removed per wk. av.	Total net Hb. output 2 wks.
gm.	*cc.*	*wks.*	*per cent*	*kg.*	*per cent*	*gm.*	*gm.*
ıd 450, salmon 100	0	2	84	14.1	42	1.6	
ıach 110, bread 283, salmon 100	0	3	80	13.1	41	14.8	
ad 363, salmon 50, meat 50	0	4	100	14.4	47	8.8	
.s 100, bread 325, salmon 50	0	2	99	13.4	52	13.5	17.5
:ad 385, salmon 50	0	2	100	13.4	45	12.9	
nach 150, salmon 50, meat 50	0	2	94	13.5	46	27.2	34.2
:ad 400, salmon 100	0	1	89	13.5	43	3.2	
bbage 125, bread 350, salmon 100	0	2	100	13.8	44	10.0	2.5
ead 425, salmon 100	0	2	100	13.9	44	8.9	
ead 394, salmon 100	40	4	94	14.2	41	3.9	
:ef kidney 160, bread 291	75	3	95	13.4	51	8.6	28.6
:ead 350, salmon 100	75	3	100	14.2	46	8.7	
iver extract,* bread 325, salmon 100	50	2	100	14.8	43	7.0	11.3
read 325, salmon 100	40	2	100	14.9	46	6.5	
e 25 mg., bread 325, salmon 100	40	4	100	15.0	52	14.3	24.1
read 325, salmon 100	40	3	100	15.0	48	6.1	
'ig liver 300, bread 300	40	2	98	13.7	60	27.2	26.0
Iread 400, salmon 100	40	3	99	13.8	46	0	
Beef muscle 375, bread 300	40	2	100	14.0	61	29.0	53.1
Bread 400, salmon 100	40	3	100	14.6	51	7.7	
Salt mixture 6, bread 400, salm. 100	40	2	100	14.4	47	7.0	0
Bread 400, salmon 100	40	2	100	14.8	46	6.5	
Salt mixture 6 + Fe 38 mg., br. 400, salm. 100	40	2	100	15.2	51	16.5	34.2
Bread 400, salmon 100	40	2	100	15.3	47	14.2	
Fe 40 mg., bread 400, salmon 100	40	2	100	14.9	52	25.2	43.6
Bread 400, salmon 100	40	2	100	15.2	41	10.3	

* Liver fraction potent in pernicious anemia—given in equivalent for 500 gm. liver daily.

† Salt mixture (McCollum and Simmonds (10)) without iron 6 gm. daily.

gional lymph nodes in large quantities after 4 hours, still demonstrable but considerably diminished after 24 hours, but no longer detectable after 48 hours. The results suggest that in mice following subcutaneous inoculation of influenza virus, the virus is absorbed in regional lymphatics and is there destroyed without invasion of the blood stream or of the lungs in contradistinction to the course of events after intraperitoneal inoculation.

Attempts to determine the effects produced by virus administered *intravenously* to mice were impeded at first by a toxic effect of suspensions of mouse lung tissue which resulted in the immediate death of most mice with symptoms of acute "pulmonary" shock. It was subsequently found that prolonged centrifugation of the material reduced its toxicity sufficiently to permit the inoculation of larger groups of mice although toxic symptoms with occasional deaths still occurred. The following experiment illustrates the results obtained.

Five mice anesthetized with ether were given into the tail veins 0.25 cc. of 5 per cent suspension of mouse lung virus in 10 per cent normal mouse serum in Locke's solution. The suspension had been previously centrifuged for 1 hour at 2,500 R.P.M., and when titrated intranasally in mice a dilution of 1:100,000 caused death. Therefore, approximately 25,000 intranasal M.L.D. were administered intravenously. Individual mice were exsanguinated from the heart after 30 minutes, and 1, 2, 4, and 6 days following inoculation. The abdominal viscera were removed at the same time. The blood was ground with 1.0 cc. of broth and 10 per cent suspensions made of liver, spleen, kidney, and lung, care being taken to avoid cross contamination of organs. Groups of 4 test mice were inoculated intranasally with 0.05 cc. amounts of each blood and organ suspension.

The results of the present experiment, summarized in Table VIII, show that following intravenous administration of large doses of virus, virus may be demonstrated consistently in the lungs, and typical influenzal pulmonary lesions may occur. Results obtained from examination of the other organs and the blood must be considered as preliminary observations. They are included, however, because of the interesting tendency of the virus to localize and persist in the liver. While no explanation can be offered at present in regard to this phenomenon, the similarity in embryological origin of the lung and liver as well as certain structural similarities might be borne in mind. It is of further interest as well that virus administered intravenously is

July 26. Bleeding continues. Calcium chloride 15 gm. given by stomach tube.

July 27. Bleeding continues. Transfusion 100 cc. normal blood. Calcium chloride 15 gm. by stomach tube. Liver 100 gm. added to diet. Food consumption good.

July 28. No bleeding, liver diet continued.

Aug. 11 and 12. Ox bile 50 cc. by stomach tube. Salmon bread diet. Calcium chloride given intermittently.

Nov. 21. Slight bleeding from venous puncture wound. Diet contains 150 gm. liver.

Nov. 28. Spontaneous bleeding from venous puncture wound. Transfusion 50 cc. normal blood. Liver increased to 300 gm. daily.

Dec. 1. Slight bleeding from venous puncture wound. Dog bile 50 cc. given by stomach tube. Bleeding continues. Transfusion 120 cc. normal blood. Beef kidney diet instead of liver.

Dec. 5 to 21. Dog bile 75 cc. added to food daily. Salmon bread diet.

Jan. 10, 1928. Sodium taurocholate 1 gm. added to daily diet.

Jan. 16. Bile omitted from diet.

Jan. 18. Dog bled about 100 cc. during the night. Sodium taurocholate omitted from diet. Dog bile 75 cc. given by stomach tube. Transfusion 130 cc. normal blood.

Jan. 19. Dog bile 50 cc. added to daily diet.

Feb. 7. Irradiated ergosterol 4 mg. daily added to food. Bile omitted.

Feb. 17. Spontaneous bleeding from venous puncture wound. Transfusion 110 cc. normal blood. Dog bile 50 cc. and calcium chloride 10 gm. by stomach tube.

Feb. 21. Dog bile 40 cc. daily added to food.

Uneventful anemia history until July 26, 1928.

July 26, 1928. Convulsions, dog appears weak—hemoglobin is 51 per cent. Transfusion 100 cc. normal blood a.m. and p.m.

July 30. Condition improved.

Dec. 7, 1927, to Aug. 20, 1928. Bile pigment output in urine per 24 hour period average. On the few occasions when transfusions were given the bile pigment figures for the 4 days subsequent to the transfusion were omitted from the averages as listed.

Dec. 7, 1927	to Jan. 7, 1928	18 mg.
Jan. 12, 1928	" Feb. 12	27 "
Feb. 15	" Mar. 15	24 "
Mar. 16	" Apr. 16	33 "
Apr. 18	" May 18	28 "
May 18	" June 18	31 "
June 20	" July 20	35 "
July 20	" Aug. 20, 1928	34 "
Average 9 months		29 mg. per 24 hours

TABLE VIII

The Distribution of Virus after Intravenous Inoculation in Mice

Organs examined	Deaths and lesions in test mice														
	30 min.			1 day			2 days			4 days			6 days		
	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
Blood	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0
Liver	+ ±	++ +	4, 4N	0 0	0 0	0 0	+ +	+++ ++	4, 6	+ ±	6, 6	4, 4	0 0	0 0	0 0
Spleen	0 0	+ 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0
Kidney	0 0	+++ ++	3, 4N	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0
Lungs	+ ±	7, 8	3, 3N	5, 6N	6, 7	—	4, 5	5, 5	—	4, 4	4, 5	—	+ +	++ ++	4, 4
Lesions seen in lungs		0			0			0			±			++	

lived a long time considering the absence of bile feeding—a period of about 15 months. The anemia period lasted 10 months and we note the reaction to liver and kidney feeding was one-half normal hemoglobin production or less. It should be noted that this dog was much smaller and of a totally different breed from that used in our standard anemia experiments. This dog was given 15 gm. hemoglobin by vein during a 2 weeks' period and showed no return in new red cell output. This is totally different from the usual reaction (4) and we have no explanation to offer.

Clinical History.—Table 5. Dog 24-9. Adult female beagle mongrel. Renal bile fistula operation done by Dr. Morton approximately Apr. 1, 1926. The dog was used by Dr. Sperry for studies in lipid excretion (8).

Sept. 25, 1926, to July 20, 1927. Experimental anemia. Routine anemia experiments. No bile nor bile salts given at any time. No transfusions.

June 2, 1927. Moderate bleeding during night from venous puncture. Stopped spontaneously.

June 22. Slight bleeding from venous puncture wound.

July 6. Slight bleeding from venous puncture wound.

July 21. Marked bleeding from two venous puncture wounds. Hematoma formed.

July 22. Slight oozing of blood still noticeable. X-ray films of lower extremities and pelvis indicate normal bone findings. Blood calcium 9 mg. per 100 cc. blood. Dog was given ether and killed. Blood clotted very slowly—40 min. No jaundice.

July 22, 1927. *Autopsy* done at once. Heart and lungs normal. *Spleen* normal in gross. Histological sections show some marrow metaplasia with many megakaryocytes in spleen pulp. Fine granules of a yellow pigment are abundant in phagocytes. Lymph glands show much of the same pigment within phagocytes in the gland pulp. *Liver* is normal in gross. Histological sections show a few granules of yellow pigment in the liver cells. A few mononuclear cells are found in the periportal stroma. The liver is essentially normal. *Fistula* is patent and the common duct occluded. There are a few silk sutures in the wall of the gall bladder and they are crusted with salts and pigment. The gall bladder wall is slightly thickened and the renal pelvis normal in gross. Histological sections show an essentially normal gall bladder mucosa. An occasional mononuclear cell is seen in the wall of the gall bladder and right renal pelvis. Small renal scars adjacent to the fistula are noted. *Kidneys:* Left kidney is slightly hypertrophied but otherwise normal. Right kidney shows some atrophy and scars. There are casts in the tubules close to the scars related to the fistula. In general the histological sections show normal glomeruli and tubules. Small *intestine* shows a deep

rapidly removed from the blood but persists in relatively high concentration in the lungs. The virus has undoubtedly been transported to the lung by way of the blood stream and concentrated there. These observations may aid in understanding the route by which virus from the peritoneal cavity reaches the lung. It is impossible at present, however, to decide whether the virus in the lung reaches its concentration merely by selective localization or to what extent it represents a result of virus multiplication.

DISCUSSION

It has been shown in the foregoing experiments that, following the intraperitoneal inoculation of mice with large doses of epidemic influenza virus, the virus reaches the lungs and may there produce macroscopic lesions of epidemic influenza virus infection. These results are obtained only when virus is administered intraperitoneally in amounts equivalent to more than 10,000 intranasal lethal doses. It is quite remarkable, however, that although as much as 100,000 M.L.D. of virus may be recovered from the lungs of mice and the virus may persist in the lungs from 4 to 10 days, pulmonary lesions of more than moderate extent have not been observed, nor have any fatalities due to virus infection occurred among the many mice subjected to the procedure. This is in striking contrast to the effects observed after intranasal inoculation of mice in which instance minute amounts of influenza virus cause a fatal disease with complete pulmonary involvement. The capacity of the virus to inflict pulmonary damage is obviously influenced by the route by which it reaches the lung, and the mechanism of its action must differ under the two conditions.

It is difficult to decide whether the virus reaches its relatively high concentration in the lung as a result of multiplication in that organ or whether it is concentrated in the lung as a result of selective localization on transport from other parts of the body. In favor of the latter hypothesis are the facts that the virus does not exert its complete, pathogenic action in the lung after intraperitoneal inoculation, and that an available source for feeding virus to the lung is in the peritoneal cells where virus has been shown to persist, if not multiply, for several days. Furthermore, virus is recovered from the lungs only after maximal intraperitoneal doses, indicating that for it to reach the

metabolism as related to hemoglobin destruction and bile pigment production. This dog's plasma volume varied from 810 to 960 cc. during this period—total blood volume from 1100 to 1250 cc. If we take the blood volume as 1100 cc. and the hemoglobin level at 50 per cent, the dog had 75.9 gm. hemoglobin in circulation. With a destruction of 0.83 per cent a day (or 0.65 gm. hemoglobin) if quantitatively changed to bile pigment (1 gm. hemoglobin = 40 mg. bile pigment) we should expect 26 mg. bile pigment per 24 hours. Likewise with a blood volume of 1200 cc. we should expect an output of 28 mg. bile pigment per 24 hours. The actual average value for this dog is 29 mg. bile pigment per 24 hours.

We must not forget the probability that the wear and tear of *muscle hemoglobin* will yield some bile pigment as muscle hemoglobin introduced into the blood promptly appears as bile pigment (9). This figure for the normal wear and tear of muscle hemoglobin is an unknown. It may even be argued that the muscle hemoglobin as a part of the matrix of the striated muscles is repaired *in situ* without any loss of the pigment radicle by waste—a rather unusual reaction in body metabolism. One may choose to believe that the slight difference between the expected bile pigment output per 24 hours of 26 to 28 mg. and the actual measured output of 29 mg. bile pigment represents a contribution from the wastage of muscle hemoglobin.

Autopsy findings in the dogs reported in this paper and others reported elsewhere (5) deserve some emphasis as they give convincing evidence that the bone marrow is structurally normal. The liver lobules are normal and there is no evidence for long continued infection or bile stasis within the biliary system of ducts. The spleen may show a little marrow metaplasia as is seen frequently in the anemia colony without fistula and reported elsewhere (7). The gastro-intestinal tract is normal histologically. Yet in spite of this normal structural picture this body machine with an established bile fistula can form only about one-half normal amounts of new hemoglobin under the stress of severe anemia (refer to Table 6).

Iron absorption obviously is seriously impaired in an anemic bile fistula dog which returns about one-half the expected hemoglobin as the result of standard doses of iron by mouth. *Iron given by vein* on the contrary will return the expected amount of new hemoglobin

lung a flooding of the body must occur. On the other hand, a mode of transport is not readily apparent, for virus has not been recovered from the blood later than 24 hours after its introduction into the peritoneal cavity. Moreover, since such large amounts of virus intraperitoneally are required to permit the detection of virus in the lung, it may be that any virus which reaches the lung can subsequently multiply. This is suggested by the fact that the highest concentration of virus in the lung is usually encountered 24 to 48 hours after virus can be recovered from the blood. At present, however, no final conclusion appears warranted.

As previously stated, mice whose lungs contain relatively high contents of influenza virus and present macroscopic lesions subsequent to the intraperitoneal injection of virus show no external evidence of infection. They do not die but become staunchly immune to infection by the intranasal route. The failure of the virus to induce fatal infection in mice under these conditions immediately suggests that the animal develops an increased resistance capable of inhibiting the pathogenicity of the virus which is abundantly present in susceptible tissues. It was shown, in fact, that mice which were given large doses of virus intraperitoneally 48 hours before the intranasal introduction of a fatal dose of virus resisted the fatal infection. The lethal intranasal dose of virus was given at the time when the highest concentration of virus in the lung is reached as a result of the intraperitoneal inoculation. It is extremely interesting that instead of lending an added effect to the intranasal dose, an antagonistic effect was observed. On the basis of the experiments recorded above, the resistance is active before demonstrable antiviral substances are present in the blood. The studies of Hoskins (6) with yellow fever virus are of interest in suggesting an interpretation of the present results. Hoskins observed that when the neurotropic strain of yellow fever virus was given to monkeys by the intraperitoneal route, the animals were rendered resistant to fatal infection by the viscerotropic strain of virus given as much as 24 hours previously. It may be in the present instance that the virus of influenza entering the pulmonary tissues *via* the peritoneal route renders the cells refractory to fatal infection with the same virus administered 48 hours later by the intranasal route.

SUMMARY

The presence of a renal bile fistula with escape of all bile into the urinary tract seriously impairs the capacity of an anemic dog to form new hemoglobin on standard diets.

These bile fistula dogs will produce about one-half as much hemoglobin in anemia on standard diets as during earlier control periods without a bile fistula.

Iron given by mouth to an anemic bile fistula dog will effect the production of about one-half the amount of new hemoglobin as in control periods.

Iron given by vein to an anemic bile fistula dog will approximate the theoretical 100 per cent return of new hemoglobin. Obviously absorption is a very important factor in the utilization of iron by these dogs.

The reaction to liver feeding is much like the reaction to iron feeding but we have no proof of inadequate protein digestion and absorption in these bile fistula dogs.

In fact the uniform body weight and normal clinical state over periods of years speak for adequate absorption of protein digestion products. Evidence cited above supports our belief that inadequate hemoglobin production (protein formation) noted in these bile fistula dogs may be related to a disturbed liver function.

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Following the same reasoning it might be suggested that the solid immunity to intranasal infection which follows intraperitoneal vaccination with active influenza virus is entirely attributable to a localization of the immunizing virus in the lungs, and that unless virus reaches and multiplies in the lungs no immunity is produced. This concept is immediately eliminated by reference to the successful vaccination of mice with inactive formalized virus (7). Furthermore, in the course of the present studies it has been possible to show that fully active immunity can be induced by the intraperitoneal injection of active virus cultivated in tissue culture medium (8, 9) although it has not been possible to detect its presence in the lungs of vaccinated mice, even after repeated passages.

SUMMARY

Following the intraperitoneal inoculation of mice with large doses of epidemic influenza virus (50,000 to 1 million intranasal M.L.D.) it can be recovered from the lungs in high concentration, and pulmonary lesions of moderate extent may be observed. The virus reaches its highest titer in the lungs 48 to 72 hours after intraperitoneal injection and may persist for 10 days. Virus may be recovered from the blood in the first 24 hours, but is readily detected in the omentum and peritoneum for 5 to 6 days. Mice which as a result of the intraperitoneal injection of virus show a high concentration of virus in the lungs do not die but become solidly immune to intranasal infection. Moreover, as early as 24 to 48 hours after intraperitoneal inoculation of large amounts of virus the animals may exhibit resistance to infection with fatal doses of virus given intranasally.

Influenza virus given intravenously to mice is rapidly removed from the blood but persists in the lungs and induces pulmonary lesions. Virus can also be recovered from the liver for several days. With subcutaneous inoculation of influenza virus, however, the virus does not reach the blood or lungs in detectable amounts although the regional lymph nodes may yield considerable quantities of the agent.

A brief consideration is presented of the mechanisms of infection and resistance which may be involved.

Methods

Dutch belted rabbits were used exclusively. They were 6 months old when received, with a few exceptions noted below. The animals were kept in individual cages indoors and fed a stock diet of oats and green vegetables. Aside from the first group reported below, each rabbit was given 1 gm. of crystalline cholesterol mixed with the moistened grain three times a week throughout the experiments.

Blood was obtained from an ear vein at weekly intervals during the preliminary feeding periods and in the intervals between experiments but more frequently during the times that the various measures were being carried out. The cholesterol was determined on the whole blood by the method of Bloor, Pelkan, and Allen (4). The same rabbit was often used for several different observations. Thus, the influence of thyroidectomy and of the administration of thyroxin, potassium iodide, and insulin with appropriate intervals between each series of observations may have been determined on a single animal.

The thyroid ablations were performed under ether anesthesia. A midline incision was made over the larynx and trachea and the glands exposed. They were then dissected out as carefully as possible to ensure complete removal. Bleeding was easily controlled by pressure. At the conclusion of the experiments an autopsy was performed and the absence of thyroid tissue verified. In 4 animals it was found that removal had been incomplete and regeneration had occurred. These animals are treated separately in the following sections.

Effect of Thyroidectomy on Blood Cholesterol of Normal Rabbits

A significant increase in the blood cholesterol of rabbits has been noted after thyroidectomy by several observers (5–9), while one investigator (10) reported that no rise occurred and another (11) that it was temporary. In a previous communication (2) it was pointed out that the general level of the blood cholesterol of a group of thyroidectomized rabbits observed for 110 days after operation was only slightly higher than that of a control group of normal animals. The average blood cholesterol of the normal group for the whole period was 110 mg. and of the thyroidectomized 121 mg.

Before going ahead with work involving the use of thyroid ablation, it seemed advisable to repeat our previous observation but this time comparing the blood cholesterol before and after operation in the same animals rather than in separate groups of animals.

The blood cholesterol was determined weekly in each of 6 rabbits for 5 weeks before and for a similar period after thyroidectomy. During this whole time the animals were kept on a normal diet without added cholesterol. The complete removal of the thyroids was verified at a later date at autopsy.

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occurred that surpassed the previously recorded high for the animal. The maximal increase during a period of 2 months after the operation varied from 74 to 255 per cent and averaged 137 per cent for the group.

The subsequent course of the blood cholesterol of these rabbits after establishing a new high varied. In general it can be said that the postoperative rise was maintained or gradually increased in 3 animals; was followed by a slight fall and then a later rise to new high levels in 2; and was temporary in 3 with a return to preoperative levels. In the last group was the animal found to have thyroid regeneration at autopsy 7 months after operation.

TABLE II

Effect of Thyroidectomy on Blood Cholesterol of Cholesterol-Fed Rabbits

Rabbit No.	Sex	Duration cholesterol feeding	Blood cholesterol, *mg. per 100 cc.*										Maximal increase
			Previous high	Preoperative level	Weeks postoperative								
					1	2	3	4	5	6	7	8	
		mos.											*per cent*
3-06	M	11	305	281	472	850	833	819	834	781	834	835	203
3-39	M	10	616	616	—	1170	1030	1560	1230	1170	1010	—	153
3-49	F	12	1170*	670	—	1080	1170	1200	902	694	738	—	79
3-51	F	6	362	358	568	675	726	610	532	400	403	521	89
3-52	M	13	762	738	—	1115	1285	1145	Died				74
3-53	M	6	333	305	397	417	385	526	500	438	556	500	82
3-56	F	13	794	690	—	1145	1065	1510	1035	705	695	—	119
3-65	M	3	232	220	782	660	638	399	430	387	361	538	255
Average													137

* Single determination; otherwise not above 736 mg.

In summary, thyroidectomy in the cholesterol-fed rabbit with hypercholesterolemia is followed by a further rise in the level of the blood cholesterol. This rise is usually maintained, but in a minority may be temporary. The magnitude of the increase is in sharp contrast to the insignificant rise that occurs following this operation in the rabbit on a normal diet without cholesterol.

Effect of Thyroidectomy on Blood Cholesterol of Resistant Rabbits

It is a common experience for anyone who has fed cholesterol to large groups of rabbits to find that certain animals do not develop a

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varied from 108 to 713 per cent with an average of 270 per cent. In no case did the blood cholesterol revert later to its previous level.

In summary, the ablation of the thyroid destroys the resistance that certain cholesterol-fed rabbits show to the development of a hypercholesterolemia. This result is of great theoretical interest.

Effect of Thyroidectomy on Response to Thyroxin

It has been well established that thyroid extract or thyroxin will lower the blood cholesterol of rabbits. This effect is particularly striking when cholesterol-fed animals with a hypercholesterolemia are used (3). There were at least two objections to this last report, however. In the first place, infrequent determinations of the cholesterol prevented a realization of the magnitude of the fall in the blood; secondly, the thyroid was fed to the rabbits concurrently with cholesterol for a 3 week period, which naturally gave less clear cut results than would be obtained by giving a single large dose of thyroxin. The present work was undertaken to correct these defects and furthermore to compare the type of response of normal and thyroidectomized animals to a single dose of thyroxin.

11 rabbits with thyroids intact were used. There were 5 males and 6 females. 3 of the animals (Nos. 2-56, 2-57, and 2-60) were 2 to 3 years old at the beginning of the feeding period; the others were the usual 6 months of age. They had been fed cholesterol for from 7 to 15 months, and all had a hypercholesterolemia that had been at a fairly constant level for the individual for weeks or months before the experiment was started. On the day the experiment was begun a control blood was taken about 9 a.m., and at 10 a.m. the rabbit was given a subcutaneous injection of 1.2 mg. of crystalline thyroxin dissolved in N 50 sodium hydroxide. Another blood was taken for cholesterol determination in the late afternoon, then twice daily for several days and with decreasing frequency thereafter. Cholesterol feeding was continued throughout the experiment.

The results are shown in Table IV. A substantial decrease in the level of the blood cholesterol occurred in each animal. The maximal drop varied from 25 to 64 per cent with an average of 40 per cent. The lowest point was usually reached on the 3rd or 4th day, but occasionally on the 2nd or 5th day. Following this drop, the cholesterol then rose steadily to its previous level which was usually attained by the 5th to 9th day but was delayed in one instance until

the end of the 3rd week. In 3 of the rabbits the original level of the blood cholesterol was exceeded during this rise. For example, No. 3-57, that had never had a blood cholesterol above 375 mg. in 9 months of feeding, showed a drop in cholesterol values to 171 mg. after thyroxin, then rose to 576 mg. on the 7th day, and had reached 938 mg. on the 15th day.

For comparison with the foregoing, a group of 11 thyroidectomized rabbits was used. 7 were males and 4 females. After 6 to 18 months of cholesterol feeding, the animals all had a hypercholesterolemia which was more marked than in the group with thyroids intact. This was due in part at least to the further rise of blood cholesterol that occurs after thyroidectomy which had been performed in these animals 3 to 10 months before. Thyroid removal was proved at autopsy to have been complete in 7 rabbits, but in 4 thyroid regeneration had taken place when an autopsy was done 1 to 3 months after the conclusion of the experiment. These animals are grouped separately in the table and listed as having had a partial thyroidectomy. Thyroxin was injected and blood cholesterol determinations made as in the intact animals.

The results are shown in Table V. As in the animals with thyroids a decrease in the blood cholesterol occurred soon after the injection of thyroxin. The maximal drop amounted to 44 to 70 per cent with an average of 61 per cent for the animals in which the thyroidectomy was complete, and was practically the same for those with an incomplete thyroidectomy, ranging from 49 to 65 per cent and averaging 60 per cent. This was a greater decrease than occurred in the rabbits with thyroid glands intact. How much this was due to an inherent difference in response between animals with and without thyroids cannot be said from this small series, but it seems probable that it was due in part at least to the higher original levels of the blood cholesterol of the thyroidectomized animals. As in the intact group, the low point of the blood cholesterol was usually reached on the 3rd or 4th day, but occurred on the 2nd day in 1 and on the 5th day in 2. The cholesterol returned to its original value in a widely variable time in 7 of the animals, and continued to higher levels in 3 of these. In 3 rabbits the blood cholesterol remained permanently below its original level.

One rabbit was given an injection of N 50 sodium hydroxide without thyroxin as a control. No change in the blood cholesterol resulted.

In summary, it seems evident that a single injection of thyroxin is capable of producing a significant drop in the blood cholesterol of rabbits with hypercholesterolemia, regardless of whether the thyroid glands are intact or have been partially or completely removed. The maximal effect is usually evident on the 3rd or 4th day after injection.

Effect of Thyroidectomy on Response to Potassium Iodide

Potassium iodide when administered in large doses concurrently with cholesterol has been shown to prevent the development of a hypercholesterolemia and the resultant formation of atheromatous lesions in the aorta (1). This protective action was found to be abolished in thyroidectomized rabbits (2), suggesting that the iodide acted in some way through the thyroid. It was later shown that when potassium iodide was given to rabbits with a high blood cholesterol due to long continued cholesterol feeding, a further increase in the hypercholesterolemia occurred (3). This result was unexpected and suggested that the iodide might be mobilizing cholesterol from the tissues, resulting in an increase in the amount in the circulating blood, but further work did not confirm this hypothesis (12). It was of interest to determine whether the action of KI in causing increase in a blood cholesterol already high was also dependent upon the presence of the thyroid, or whether, possibly, some other mechanism was involved.

6 intact rabbits with a hypercholesterolemia due to cholesterol feeding of from 9 to 19 months' duration were each given a gram of potassium iodide as an aqueous solution mixed with the grain three times a week for 2 weeks. KI was then discontinued but the cholesterol feeding was carried on as usual.

The results are shown in Table VI. As had been found before (3), a definite rise in the level of the blood cholesterol occurred in 5 of the 6 animals. With the exception of the one animal whose blood was unchanged, the average increase for the group was 44 per cent, ranging from 14 to 74 per cent. For the entire group of 6 rabbits the increase averaged 37 per cent. The rise tended to occur in an irregular fashion and a definite peak was often not demonstrable. This type of effect is in sharp contrast to that of thyroxin.

For comparison with these rabbits with intact thyroids, there were 10 animals shown later at postmortem examination to have been completely thyroidectomized and 4 in which the thyroidectomy was incomplete. In this total group of 14 were 9 males and 5 females. When the experiment was begun, the animals had been fed cholesterol for a period of 3 to 16 months, and had been thyroidectomized 1 to 8 months. Cholesterol feeding was continued throughout. Each rabbit was given 1 gm. of potassium iodide three times a week for 2 weeks.

The results are shown in Table VII. All the rabbits responded to the potassium iodide feeding with an increase in their existing hypercholesterolemia amounting to from 34 to 122 per cent. The average for those rabbits having no thyroid tissue was 85 per cent, and for those in which thyroidectomy had been incomplete it was 50 per cent. The peak of the increase in the completely thyroidectomized group came usually in the 4th week. In the partially thyroidectomized animals the maximal increase occurred in 2 or 3 weeks.

In summary, the action of potassium iodide in causing a further increase in hypercholesterolemia of cholesterol-fed rabbits is not abolished by thyroidectomy. This is in sharp contrast to the effect of potassium iodide in preventing a rise of blood cholesterol when the drug is given at the beginning of the cholesterol feeding, an effect that is abolished by thyroidectomy. Obviously two different mechanisms are involved.

Effect of Thyroidectomy on Response to Insulin

Because the effect of insulin in lowering blood sugar occurs promptly after injection, it seemed likely that, if there were a concurrent effect on blood cholesterol, this should be manifest in hours rather than in days.

The influence of insulin on blood cholesterol was tested in rabbits with and without thyroids.

The plan followed was to secure a control reading of the cholesterol and sugar in the blood of the animal before injecting insulin and to repeat this determination 2, 4, and 6 hours after the injection. In addition, the blood cholesterol was determined on the 2 days following the insulin. Each rabbit regardless of weight was given 3 units of insulin. From the time the control blood was taken until the sample was obtained 6 hours after insulin the animal was given no food or water.

As a control to the possible effects of insulin injection the diurnal variation in blood cholesterol of 5 fasting rabbits was determined. The results are shown in

TABLE VIII

Diurnal Variation in Blood Cholesterol

Rabbit No.	Blood cholesterol, *mg. per 100 cc.*					Maximal variation
	9.00 a.m.	11.00 a.m.	1.00 p.m.	3.00 p.m.	5.00 p.m.	*per cent*
1-87(*a*)	120	107	115	113	110	6
(*b*)	105	105	109	111	116	6
1-93	442	427	437	418	422	3
1-98	124	120	122	116	124	4
1-92	441	431	412	417	452	5
2-31	99	105	99	100	100	4

TABLE IX

Effect of Insulin on Hypercholesterolemia of Intact Rabbits

Rabbit No.		Blood, *mg. per 100 cc.*							Maximal decrease
		Days							
		1					2	3	
		9.00 a.m.	10.00 a.m.	12.00 m.	2.00 p.m.	4.00 p.m.			*per cent*
3-08	Cholesterol	700	3 units of insulin injected	658	682	694	568	910	19
	Sugar	107		56	119	113	—	—	
3-36	Cholesterol	528		646	577	586	609	762	Rise
	Sugar	104		62	105	127	—	—	
3-40	Cholesterol	958		860	860	902	948	950	10
	Sugar	108		87	113	107	—	—	
3-47	Cholesterol	560		595	544	560	636	638	3
	Sugar	91		28	66	104	—	—	
3-61	Cholesterol	738		736	636	614	656	782	17
	Sugar	112		20	79	125	—	—	
3-68	Cholesterol	762		636	646	646	710	882	17
	Sugar	118		42	81	112	—	—	
3-69	Cholesterol	837		727	658	815	694	853	21
	Sugar	101		29	49	94			
Average									15

Table VIII. In 3 animals with a blood cholesterol at normal levels the maximal variation was from 6 to 13 mg. In 2 rabbits with hypercholesterolemia the variation was proportionally no greater. All figures were within the limits of accuracy for the method.

When intact rabbits in possession of their thyroids were injected with insulin a prompt decrease in blood sugar occurred as might be expected. There was also a tendency for the blood cholesterol to fall (Table IX). A decrease amounting to 10 to 21 per cent occurred in 5 of 7 rabbits. The drop in the cholesterol was maximal in 2 hours in 2, and at 4 hours, 6 hours, and 24 hours in the remaining 3. There was no definite correlation between the degree of fall in the blood sugar and blood cholesterol.

For comparison with this group of intact rabbits, 6 animals with thyroids completely removed and 4 partially thyroidectomized were used. As will be noted in Table X a decrease in both the blood sugar and blood cholesterol occurred. Not only did thyroidectomy fail to interfere with this reaction, but, on the contrary, the fluctuations were even more pronounced. 3 of the rabbits developed hypoglycemia convulsions which were fatal in one instance. The blood cholesterol decreased 10 to 33 per cent in each case. In half the rabbits the drop was maximal at 6 hours, while in 2 others the lowest point in the curve was reached at 4 hours.

In summary, insulin causes a decrease in blood cholesterol in rabbits with or without thyroid glands. The fall is somewhat more marked in the absence of the thyroid.

SUMMARY

1. The blood cholesterol of rabbits on a normal diet without added cholesterol is increased only slightly (19 per cent) by thyroidectomy.

2. In rabbits with a hypercholesterolemia due to long continued cholesterol feeding, thyroidectomy causes a marked rise (137 per cent) in the blood cholesterol. This rise is usually maintained.

3. When long continued cholesterol feeding has failed to cause a rise in the blood cholesterol of rabbits, thyroidectomy abolishes this resistance and a hypercholesterolemia is promptly produced.

4. A single injection of thyroxin causes a significant drop in the blood cholesterol of rabbits with hypercholesterolemia. This reaction is not influenced by thyroidectomy.

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ence in exudates would offer a reasonable explanation for the increased passage of fluid from the circulating blood into an inflamed area.

Observations, to be described, indicate that the active factor is a dialyzable, non-protein, nitrogenous crystalline substance. The present evidence points to its being an intermediary product of protein catabolism probably related to the group of simple polypeptides. In an accompanying paper it will be shown that this substance seems to be likewise concerned with the mechanism of leukocytic migration in inflammatory foci.

Purification of the Factor

Exudates were generally obtained, as described previously, from the pleural cavities of dogs after intrapleural injection with 1 to 2 cc. of turpentine (2). The exudate resulting from the intraperitoneal injection of an aleuronat-starch mixture in a rabbit was likewise studied.

The cell-free exudate was treated with an equal volume of pyridine. A heavy flocculating precipitate resulted. The mixture was vigorously shaken for several hours in an electrical stirring apparatus. The material was then centrifugalized at rapid speed. The supernatant layer appeared as a yellowish green fluid, occasionally tinted reddish brown. This fraction, when evaporated *in vacuo* at about 45°C. yielded, upon resuspension in saline or distilled water, a substance that readily enhanced normal capillary permeability. Blood serum similarly treated yielded a material that was practically inactive.

Further purification of the active factor was attained by treating the supernatant-pyridine fraction with an equal volume of acetone.[1] The resulting heavy greyish precipitate was separated by centrifugalization. The active factor was found primarily in the acetone supernatant fraction. Storing it at 6°C. or preferably at −20°C. for several days, favored the separation of a brownish, flocculating mass. This substance, taken up in distilled water and injected intracutaneously in a rabbit, increased the permeability of skin capillaries, as indicated by the usual test (2).

The acetone supernatant fraction can also be evaporated *in vacuo* at about 50°C. The residue consists of a heterogeneous crystalline mass in which one encounters a variable amount of amorphous tarry material. The crystalline material consists of large branch-like crystals, structurally characterized by the presence of a main shaft with spicules projecting laterally on either side. Close,

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[1] In earlier studies, as described elsewhere (4), the pyridine supernatant fraction was treated with three times its volume of acetone. Subsequently, however, it was found that a significant part of the active material was apparently lost through this procedure. The use of an equal volume of acetone seemed to yield distinctly better results and this concentration was therefore resorted to in all subsequent extractions.

2. The active factor can be dissociated so as to be relatively free of sodium chloride admixture by evaporating the acetone supernatant fraction (Table I) at room temperature. Exposing the active material *in vacuo*, at relatively low temperature, yields after a variable interval a dark brownish syrupy material localized in the center of the watch glass. The periphery of the evaporated mother liquor reveals deposits of branched crystals. The viscous brown fluid in the center is pipetted off into another watch glass and dried *in vacuo* at slightly elevated temperature. The end-product is principally made up of a large number of small discrete aggregates of doubly refractive granules. In some preparations there separates out a number of elongated needle-like crystals, some of which may cohere and thus assume a rosette-like appearance. The great majority of these, however, tend to be discrete. There are none of the typical branch-like crystals. The chloride reaction with silver nitrate, if at all present, is barely perceptible. The material is very potent in the skin, producing dilatation of capillaries and lymphatics. Trypan blue from the circulating blood stream permeates in abundance into such treated areas and, as will be pointed out in the accompanying report, this is followed by prompt leukocytic infiltration.

3. Dakin demonstrated a number of years ago that, by the use of butyl alcohol as a solvent, certain products of protein hydrolysis may readily be extracted (8). These included the monoamino acids, proline, and the peptide-anhydrides (diketopiperazines). The latter could be conveniently separated from proline by their sparing solubility in alcohol or water. This almost specific extracting capacity of butyl alcohol for intermediary products of proteolytic breakdown was utilized in an endeavor to further purify the permeability factor.

The procedure adopted was as follows: The acetone-dried residue of an exudate was obtained by evaporating *in vacuo* 50 cc. of the supernatant acetone fraction (Table I). This material was taken up with 25 cc. of butyl alcohol. The resulting solution was tinted yellow and contained a suspension of fine particles. The active factor was extracted with butyl alcohol by continuous stirring for several hours. A considerable part of the solution evaporated off during the period of extraction. A cream-colored sediment settled at the bottom of the container; the sides of the vessel above the supernatant layer became coated with a crystalline granular material. The solution was centrifugalized to dispose of any suspended granules in the light amber-colored superficial layer. This clear fraction was then carefully pipetted into a watch crystal and evaporated *in vacuo* at about 50°C. The dried material revealed the presence of discrete crystalline aggregates,

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solution of picric acid yields, after several days in the refrigerator at about 6°C., a flocculating crystalline material. These picrate crystals, filtered from their mother liquor, appear at first as small clusters of tiny globular, doubly refractive crystalline material. After further drying, discrete needle-like crystals may separate out. This crystalline material induces in the skin both an increased capillary permeability and, as will be pointed out in the accompanying paper, an active migration of leukocytes.

characterized by small clusters of doubly refractive granules. These appeared as if they were bound together by the presence of an ill defined, greyish, homogeneous matrix (Fig. 1). The crystalline material formed a cloudy suspension in water. When the latter was allowed to dry gradually in a desiccator, beautiful needle-like crystals often separated out (Fig. 2).[3]

The crystals, obtained by all three of the methods outlined, appeared to be of similar structure. This was characterized by the formation of discrete aggregates of doubly refractive granules, of needle-like or small rod-like crystals, or of a mixture of both types of crystalline material. The similarity of the end-product attained by different analytical procedures strongly suggests that one is dealing with the same active substance. The crystalline material is extremely active in inducing in treated cutaneous areas of rabbits both increased capillary permeability and leukocytic migration (5). The test for chloride impurities is essentially negative. The sodium content is considerably reduced (6.4 per cent). The available evidence indicates that this crystalline substance is in all probability the active permeability factor present in inflammatory exudates, isolated in a form which seems to be relatively free of any gross impurities.[4]

The foregoing procedures have been employed in the further purification of the active material. The butyl alcohol method and the separation at −20°C. from the acetone fraction seem to yield the most effective results. The various schemes of extraction employed are summarized in their main features in Table I.[5]

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[3] The brownish sediment which settles after butyl alcohol treatment may likewise be active in inducing both increased capillary permeability and leukocytic migration. It contains, however, considerable inorganic impurities such as chlorides.

Furthermore, subjecting the butyl alcohol supernatant fraction for several days at −20°C. favors the separation of a fine granular material which *per se* is usually very effective in increasing capillary permeability and in causing leukocytic migration.

[4] Present day methods for testing the chemical purity of proteins or presumably of their derivatives are, as recently pointed out by Northrop, not very satisfactory (11). Studies are now in progress to test the purity of the isolated active permeability factor by applying some of the criteria adopted by Northrop and Kunitz in their own studies.

[5] Repeated treatment with methyl alcohol has not been found to dispose of the excess sodium chloride, inasmuch as the alcohol readily dissolves the combined

The contact of this substance for several hours with normal hydrochloric acid does not seem to affect its potency. Similar treatment, however, with normal sodium hydroxide inactivates the material.

4. *Effect of Heat or Cold.*—The material is thermostable. When brought to 100°C., it is found still active. When a test tube containing it is placed in boiling water for 15 to 20 minutes, it displays marked activity upon its subsequent intracutaneous inoculation. Heating it *in vacuo* at 85°C. for 11 hours has failed to reduce its activity. In a similar fashion, when exposed even for several days at −20°C., the material retains full potency.

5. *Melting Point.*—In its present stage of purification the active material fails to show a sharp melting point. From about 200°C. or even somewhat below this temperature, to 250°C. it begins to char; and at 300°C. it still has not melted. It is well known that a number of proteolytic compounds behave in similar fashion. Greenstein (9), in his recent studies on peptide synthesis, described a similar behavior in the case of the crystalline bisanhydro-*l*-cystinyl-*l*-cystine.

6. *Diffusibility.*—In an earlier communication, it was pointed out that the active permeability factor of exudates readily diffuses through a cellophane membrane (2). As pointed out above, the supernatant acetone fraction containing the active substance may be dialyzed for several hours against distilled water (Table I). Evaporation of both the dialyzed and the dialysate or diffusate fractions reveals the presence of the active crystalline material.

7. *Precipitation with Ammonium Sulfate.*—The active permeability factor is carried down with the precipitate that forms by the interaction of the exudate with saturated ammonium sulfate (2). Likewise, the addition of the relatively pure fraction of the material to a saturated solution of ammonium sulfate is soon followed by the appearance of a small but definite cloudy suspension. It is interesting to note in this connection that, of the dialyzable proteolytic breakdown products, polypeptides seem to be the only ones that are precipitated by concentrated ammonium sulfate (10).

An attempt was made to determine the effect on the active substance of precipitating it with saturated ammonium sulfate at differing pH values. It is to be recalled in this connection that Northrop recently succeeded in purifying crystalline enzymes in this way (11). The technique employed by us was as follows:

30 mg. or less of active material obtained in the form of the acetone-dried residue from an exudate (Table I) was treated with 1 cc. of a phosphate buffer solution at various pH concentrations as follows: 5.3, 6.8, 7.3, and 7.8. The active material seemed to enter more readily into solution at buffer mixtures of lower pH. To each sample, 1 cc. of saturated $(NH_4)_2SO_4$ was added. A cloudy suspension resulted which seemed to be somewhat more prominent in the acid media. All the fractions were kept on ice overnight. On the next day, a coarse, brown, doubly refractive precipitate was found to have formed in each of the samples.

The precipitates were separated, taken up in distilled water, and injected intracutaneously in rabbits to test for their effects on the permeability of small vessels.

method of Folin (12) for the determination of amino nitrogen was employed throughout. About 0.2 to 0.5 cc. of exudate and of serum

TABLE II

Correlation between Amino Acid Level in Serum and Exudate and the Effect of the Latter on Capillary Permeability

Dog. No.	Duration of inflammation	Amino acid nitrogen		Accumulation of trypan blue in skin areas treated with	
		Blood serum	Exudate	Blood serum*	Exudate
	days	*mg. per 100 cc.*	*mg. per 100 cc.*		
1-04	1	5.35	6.4	+	++
	2	5.3	6.5	Trace	++
	3	2.9	3.3	0	+
	4	3.75	4.75	+	+++
Reinjected 1.5 cc. turpentine on 6th day					
	7	6.05	8.9	+ to ++	++++
	8	5.75	12.6	+	+++
	9	5.75	10.3	Faint trace	+++
	10	6.15	20.95	+	++++
1-05	1	7.8	7.6	Trace	Trace to +
	2	6.15	6.4	+	+
Reinjected 1.5 cc. turpentine					
	6	4.0	6.05	0	++++
	8	5.25	6.05	+	++
	9	4.3	5.35	+	+
1-06	1	7.6	6.1	+	+
	4	3.6	4.05	Trace	+ to ++
	5	8.2	12.25	Trace to +	+
1-03	5	6.4	9.2	0	++
Average..........		5.55	8.04		

* Cutaneous areas inoculated with serum invariably stained only the peripheral portion of the areas.

was injected each in the dermis of the abdomen of a rabbit. This was followed by the intravenous injection of trypan blue to determine the effect of these samples on the capillary wall. The results

amount of nitrogen was recovered, averaging 8.65 per cent (Table III).[6]

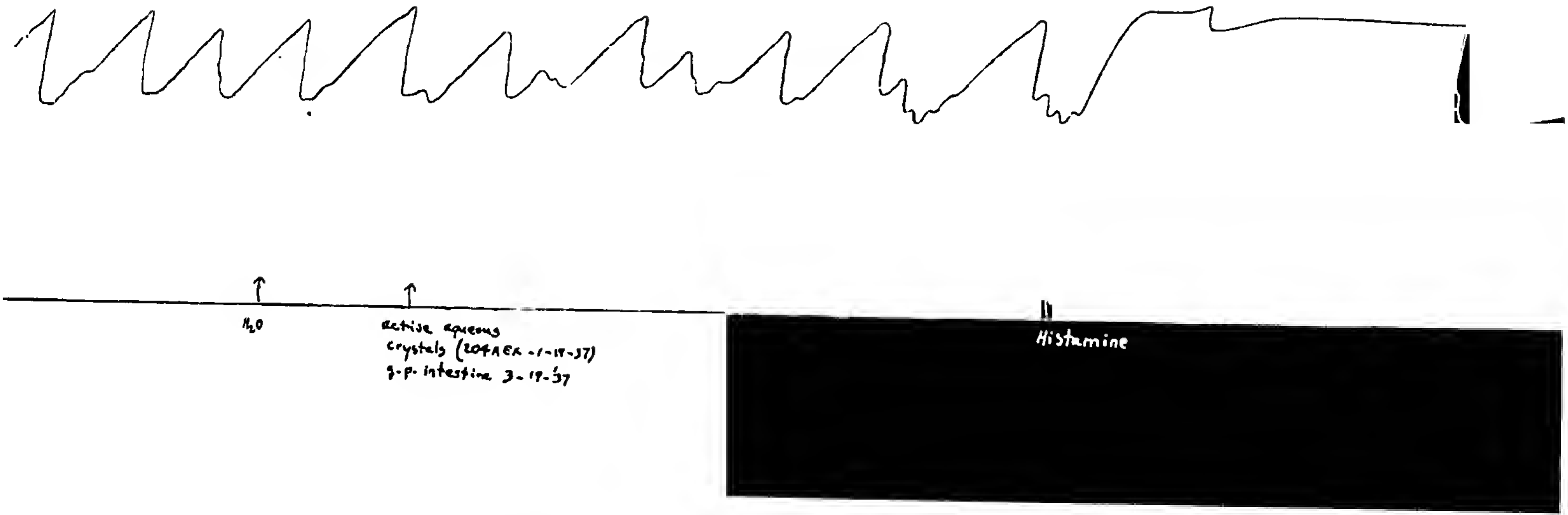
In conclusion, the various tests outlined above, in addition to the apparent zwitterionic or dipolar ionic property of the permeability factor, the capacity of saturated ammonium sulfate and of 5 per cent phosphomolybdic acid (16) to precipitate it, and finally its diffusibility through a cellophane membrane, all suggest that the active crystalline substance may well be a relatively simple intermediary product of protein catabolism. It appears unlikely that it is a proteose, a peptone, or an amino acid. The available data seem to indicate that this active substance probably belongs to the group of polypeptides. In a separate report observations will be presented indicating that a proteolytic enzyme, incubated with an otherwise relatively inactive blood serum, favors the formation of products that increase vascular permeability. These findings offer an additional

TABLE III

Protein Nitrogen of Inflammatory Exudates

Dog. No.	Duration of inflammation	Protein		Nitrogen content of active crystalline substance recovered from an exudate
		Blood serum	Exudate	
	days	*gm. per 100 cc.*	*gm. per 100 cc.*	*gm. per 100 cc.*
6-3	1	—	4.6	
	2	5.0	4.0	
6-2	1	6.4	5.1	8.1
	2	—	4.7	9.2
	3	—	5.4	
	4	6.3	5.5	
6-1	1	—	4.3	
	2	—	4.4	
	4	6.7	—	
Average.....		6.1	4.75	8.65

[6] In preliminary publication (4) the nitrogenous content was reported to average 2.3 per cent. This determination had been made on the active acetone-dried residue consisting of inorganic impurities with which the active material was probably associated. Further purification of the material by butyl alcohol has yielded correspondingly higher nitrogenous values.



TEXT-FIG. 1. Effect of the addition to the isolated strip of guinea pig intestine of the active crystalline material recovered from an exudate after preliminary treatment with pyridine and acetone. This was followed by the addition of histamine (diluted about 1:10,000) as indicated. The amount of the diluted factor added was still within the range which induces increased capillary permeability. Compare, on the other hand, the failure of the active crystalline material to induce increased contractility with the powerfully sustained contraction elicited by histamine.

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EXPLANATION OF PLATE 3

FIG. 1. High power drawing (about ×760) of aggregates of doubly refractile granules recovered from an exudate after preliminary treatment with pyridine, acetone, and final purification with butyl alcohol (Table I). This material, taken up in distilled water and injected intracutaneously, induces increased capillary permeability and leukocytic migration.

FIG. 2. High power drawing (about ×760) of needle-like crystals of the permeability factor. These crystals were obtained by gradually drying in a desiccator small amounts of the aqueous suspension of the material recovered after butyl alcohol purification (Fig. 1). These crystals taken up in distilled water, induce prompt increased capillary permeability followed by considerable leukocytic migration.

(the so called negative chemotaxis). Dead or living cultures of bacteria, on the other hand, were found to possess strong positive chemotaxis. Buchner (6) reported the finding of a protein from the Friedländer organism, capable of exerting a strong chemotactic influence. He found that glycine and leucine were definitely chemotactic whereas tyrosine and trimethylamine failed to attract leukocytes. Wolf studied the phenomena of chemotaxis *in vitro* (7). She found that the calcium ion was the only inorganic ion which *per se* was positively chemotactic. She reported that, to a certain extent, all amino acids and amines are likewise positively chemotactic.

The present observations describe the chemotactic effect of the crystalline nitrogenous substance recovered from inflammatory exudates, and which is evidently not the irritant itself (9).[1] The attraction for leukocytes is exhibited by the untreated cell-free exudate as well, indicating thus that the effect elicited by the purified substance is not likely to be due to some by-product formed during the process of chemical extraction. The observations suggest that the active crystalline substance may well be a chemotactic substance liberated by injured tissue such, for instance, as was originally postulated to exist by Massart and Bordet in 1891. This does not preclude the fact that certain irritants may not themselves contain this substance. Their injection would doubtless hasten the process of cell migration.

EXPERIMENTAL

The method of obtaining exudates by the intrapleural injection of turpentine has previously been described (9). The purification of the permeability factor from exudates has also been reported in a separate communication (1). 0.2 to 0.5 cc. of untreated cell-free exudate, or small amounts of the active material in distilled water, saline, blood serum, or the crystalline material extracted from normal blood serum were introduced into the skin of the same animal. 20 minutes to 1 hour later the involved areas, as well as normal skin, were excised, fixed in 10 per cent formalin, and sectioned.[2]

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[1] The chemical extraction of turpentine, the irritant utilized in most of the experiments, by the method employed for the analysis of exudates (1) failed to yield the active crystalline substance. Furthermore, as previously pointed out (9), the permeability factor is present in exudates resulting from a burn, indicating that the introduction of a chemical irritant is unnecessary.

[2] In the majority of the experiments, prior to excision of the skin areas, dilute trypan blue solution was injected intravenously to determine the effect of the various fractions on the endothelial permeability (1). The passage of dye into

Careful microscopic examination of control cutaneous areas essentially failed to show any evidence of leukocytic *migration through* the endothelial wall of capillaries or venules. Only occasionally an isolated cell was observed adhering closely to the wall of the vascular lumen (Fig. 1). A totally different state of affairs presented itself when the active crystalline material in either distilled water or saline was intracutaneously injected. Within 20 minutes or even at times earlier, there was a *conspicuous aggregation* of polymorphonuclear cells filling the lumens of the involved cutaneous capillaries. The cells collected peripherally, adhering closely to the endothelial wall. Within 40 minutes to 1 hour the perivascular areas displayed a moderate to considerable infiltration of polymorphonuclear leukocytes (Figs. 2, 3, and 4).

Microscopic examination revealed marked edema as indicated by the separation of collagenous bundles. The capillaries were dilated and in many instances definitely engorged. At times, a delicate fibrinous reticulum partly occluded some of the lymphatic vessels, although as a rule these structures were merely dilated and unoccluded. Diapedesis of red cells was occasionally observed, but this was not an invariable feature of the reaction. From some preliminary quantitative tests it seems as if the threshold concentration of the active substance necessary for the occurrence of cellular migration is several times that required for the detection of increased capillary permeability (1). This probably indicates that the alteration in the endothelial membrane is the initial reaction. When, however, the critical concentration of the active crystalline substance is reached (about 1:10,000 to 1:25,000), this is followed by cellular migration.

The obvious question arises as to whether cellular migration is induced directly by the presence of the material or whether it represents a secondary effect following the initial increase in capillary permeability, which in turn has been shown to be primarily referable to the crystalline substance (1). Although this question cannot be fully answered as yet, there is strong evidence to suggest that the active

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treated cutaneous areas evidently failed to influence cell migration in the interval studied, as indicated by controlled observations with various irritants such as aleuronat and turpentine.

material *per se* is capable of calling forth polymorphonuclear leukocytes. Turpentine injected into the skin induces an almost immediate augmentation in the permeability of the small vessels. Trypan blue accumulates readily from the circulating blood several minutes after the cutaneous injection of this irritant. The involved skin areas become deeply stained with the dye. About 40 minutes after the cutaneous injection of turpentine, microscopic section of the treated areas reveals marked dilatation and congestion of the small vessels with swelling of the endothelial cells. There is, however, no evidence of any aggregation and migratory activity on the part of polymorphonuclear cells (Fig. 5). This indicates that perhaps turpentine acts directly on the endothelial wall and thus by sufficient injury of the membrane favors profuse seepage of plasma into the perivascular tissue. It is conceivable, however, that the critical concentration of the active material is not attained 40 minutes or so subsequent to the injection of turpentine. At any rate, this observation indicates that a prompt and extensive increase in capillary permeability produced by a powerful irritant is not necessarily followed by the same rapid cell migration as is observed in areas treated with the crystalline substance. Results similar to those obtained with turpentine were noted with the use of aleuronat as an irritant. It is difficult in view of these findings to assume that cell migration is primarily related to the alteration in the permeability of the endothelial membrane. It seems more reasonable, at least on the basis of the present observed facts, to refer the migration of leukocytes directly rather than indirectly to the presence of the active crystalline substance. The rapidity of cellular migration induced by the injection of the permeability factor tends to favor such an interpretation. The possibility, however, of an accelerated reaction initiated by the factor which in turn favors the prompt secondary release of a chemotactic substance cannot as yet be fully ruled out.

There is further evidence which indicates that this material *per se* exerts a definite chemotactic influence on leukocytes. This is exemplified by the following observations obtained by a technique that had frequently been employed in the earlier studies on chemotaxis (4).

The substance either in saline or in distilled water was introduced into glass capillary tubes. Each of the latter was then sealed at one end and introduced, under ether anesthesia, into the peritoneal cavity of a rabbit. This animal had,

several hours earlier, been intraperitoneally injected with a mixture of aleuronat and starch. After a lapse of several hours, it was sacrificed and the capillary tubes examined for the presence of leukocytes. The tubes were usually found to be swarming with leukocytes. These cells had migrated a considerable distance inward. In the capillary sample containing either saline, distilled water, or the extracted inactive crystalline material from normal blood serum attraction of leukocytes (as measured by the extent of migration), athough present, was as a rule distinctly less marked than in the tubes containing the active crystalline substance.

Lactic acid in a capillary glass tube failed to attract any leukocytes, thus confirming the negative chemotactic tendency of this substance described by earlier writers.

The observations indicate that the substance *per se* is positively chemotactic for leukocytes.[3]

DISCUSSION

The evidences brought forward in the preceding and present communications of this series indicate the presence of both a permeability and a chemotactic factor. Are these two factors referable to one and the same substance? Two main considerations seem to support such a view.

1. The untreated inflammatory exudate contains both factors. Prolonged chemical fractionation yields a relatively homogeneous crystalline end-product which still manifests both of the properties possessed by the original exudate. The analytical procedure has thus failed to dissociate the two factors. Although this type of evidence is strongly suggestive of a single substance, it is, however, to be borne in mind that only the certitude of complete chemical purity of the material can establish this as a definite fact.

2. Is leukocytic migration referable to the initial increased capillary permeability or do the evidences on hand point to a direct effect by the active crystalline substance? The latter interpretation seems to be favored, in view of the observations cited above with the use of strong inflammatory irritants, such as turpentine or aleuronat. These substances are capable of inducing prompt filtration through the en-

[3] Another type of evidence supporting the view that the substance in question is *per se* positively chemotactic for leukocytes was obtained by observing on a slide, within a relatively short time, the collection and clustering of supravitally stained polymorphonuclear leukocytes from an exudate around particles of the active material.

dothelial wall without at the same time inducing as rapid leukocytic migration as is obtained with the active material recovered from exudates. The chemotactic property of the active crystalline substance is also in accord with the view of a direct effect favoring the outward migration of polymorphonuclear cells.

That the threshold concentrations of the active substance for the detection of increased capillary permeability and of leukocytic migration are not at the same level does not necessarily contravert the contention of a single substance. Two distinct manifestations elicited by a single substance may very well occur at different threshold concentrations.

In brief, the same two factors possessed by the untreated exudates have likewise been recovered in a crystalline substance isolated from the same exudates. This active substance appears to belong to the group of simple polypeptides.[4] The active crystalline material is thus capable of rapidly reproducing the basic sequences of the inflammatory reaction, namely an initial increased capillary permeability followed by polymorphonuclear leukocytic infiltration. For the sake of convenience it is believed that the available data warrant a name for this active substance which *per se* appears to behave as a definite biological unit. Leukotaxine has therefore been tentatively proposed for this active crystalline material recovered from exudates (2).

The precise mechanism of cell migration into an area of injury is still problematic. The lowering in surface tension hypothesis (8) or the recent explanation advanced by Abramson (10) referring cellular migration to differences in electrical potential between injured tissue and the charge on leukocytes, both constitute interesting views. Unfortunately at the present stage these hypotheses appear, on rigid scrutiny, to be largely the outcome of reasoning by analogy and are, therefore, primarily speculative in nature. It is conceivable that the isolation of an active substance from injured tissue, with chemotactic properties, may provide a new and more concrete weapon for the

[4] Other polypeptides (*e.g.* contained in glutathione) or amino acids (*e.g.* glycine and tryptophane) are incapable of inducing both the rapidly increased capillary permeability and migration of cells as elicited in dilute concentration by the active substance. These comparative studies with polypeptides or amino acids represent merely preliminary observations, which will be reported *in extenso* in a future communication.

solution of this basic problem. Further studies are now in progress in an effort to determine the exact nature of the mechanism involved.

The available evidence suggests that the presence of an irritant in normal tissue interferes with local protein metabolism (1). The result is the accumulation of incompletely hydrolyzed products of protein catabolism. One of these split or intermediary compounds is possibly leukotaxine. This active substance appears to initiate the primary sequences of inflammation, *i.e.* the increase in capillary permeability followed by the adherence and migration of polymorphonuclear leukocytes through the endothelial wall. It is conceivable that tissue injury is primarily referable to a common denominator, namely the release *in situ* of leukotaxine. The degree of injury may thus be a function of its concentration.

Is there any other regulating factor present in an inflamed area which determines the extent of cellular migration? As indicated in previous studies, there develops in an area of inflammation an increased glycolysis (11). With the depletion in the alkali reserve a true local acidosis ensues which is referable to the enhanced formation of lactic acid. When the pH of the exudate falls to a level ranging between 7.0 and 6.8, the cytological picture is characterized by a predominance of mononuclear phagocytes. At higher pH, as found in the initial stage of the inflammatory reaction, the polymorphonuclear cells constitute almost the only cytological element (12). Intensive migration of cells through the capillary wall occurs as a rule in the earlier phase of an inflammatory reaction. It is conceivable that the relative absence or reduction in cell migration in the later stages of inflammation, may in part be referable to the local accumulation of lactic acid which is in itself negatively chemotactic (5). The presence of this acid would counteract the initial chemotactic influence of leukotaxine.

SUMMARY AND CONCLUSIONS

A crystalline nitrogenous substance recovered from inflammatory exudates induces in cutaneous tissue a rapid migration of polymorphonuclear cells through the endothelial wall of small vessels. For the sake of convenience the tentative name of leukotaxine has been proposed for this active substance which *per se* is capable of rapidly reproducing the basic sequences of the inflammatory reaction, namely,

the initial increased capillary permeability followed by the outward migration of polymorphonuclear leukocytes. Comparison of the effect of various inflammatory irritants (*e.g.* turpentine or aleuronat) with that of leukotaxine indicates that the migration of cells does not seem to be primarily referable to the initial increase in vascular permeation. Furthermore leukotaxine is definitely chemotactic. It induces an active migration of leukocytes into capillary glass tubes containing this substance.

The relation of leukotaxine to the mechanism of cellular migration at the site of inflammation is pointed out.

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EXPLANATION OF PLATES

PLATE 4

FIG. 1. Capillary vessel in a cutaneous area of the dermis of a rabbit (16-73) 1 hour and 17 minutes after the local injection of physiological saline. Note the absence of leukocytic migration. ×600 approximately.

FIG. 2. Dilated capillary vessel in a cutaneous area of the same rabbit as in Fig. 1, 1 hour and 19 minutes after the local injection of leukotaxine in saline. This material, recovered from an exudate, had been partially purified by pyridine and acetone treatment. The extensive aggregation and migration of polymorphonuclear leukocytes into the perivascular tissue is noteworthy. The endothelial cells are markedly swollen. ×600 approximately.

FIG. 3. Adherence to the endothelial wall and pronounced migration of polymorphonuclear leukocytes. A dilated cutaneous vessel about 30 to 40 minutes after the intradermal injection of an aqueous solution of partially purified leukotaxine recovered from an exudate (rabbit 16-48). ×420 approximately.

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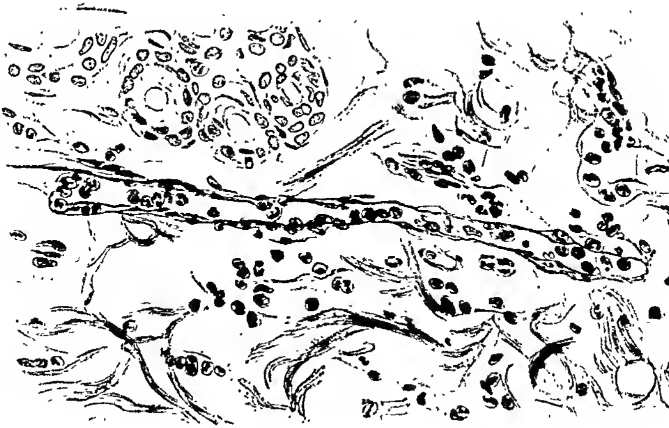
PLATE 4

1

2



3

(Menkin: Studies on inflammation. XV)

PLATE 5

FIG. 4. Cutaneous vessels 41 minutes after the intradermal inoculation of relatively pure leukotaxine (butyl alcohol purification). The migration and perivascular infiltration of polymorphonuclear leukocytes are striking (rabbit 17-03). $\times$ 680 approximately.

FIG. 5. Effect of a strong non-specific irritant on cellular migration. Turpentine was injected into the skin of a rabbit (16-66). Intravenously injected trypan blue accumulated almost immediately into such a treated cutaneous area. 38 minutes later the area was fixed and sectioned. Note that the small vessels are moderately dilated, extremely congested, and that some of the endothelial cells are a trifle swollen. There is, however, in the time interval of the experiment, no evidence of any aggregation or migration of polymorphonuclear leukocytes. This is in striking contrast to the effect induced by leukotaxine in similar intervals (Figs. 3 and 4). $\times$ 680 approximately.

4

5

STUDIES ON INFLAMMATION

XVI. On the Formation of a Chemotactic Substance by Enzymatic Action*

By VALY MENKIN, M.D.

(*From the Department of Pathology, The Harvard Medical School, Boston*)

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In earlier communications observations were presented indicating the presence in inflammatory exudates of a crystalline nitrogenous substance capable of inducing increased capillary permeability and active migration of leukocytes through the endothelial wall. In view of its chemotactic properties this substance was named leukotaxine. The accumulated body of evidence suggested that it represented an intermediary product of protein catabolism, belonging probably to the group of relatively simple polypeptides (1–5). The purpose of this short paper is to present further observations in support of this concept. Normal blood serum contains a negligible quantity of leukotaxine. Subjected, however, for a limited interval to the action of proteolytic enzymes, for example trypsin, blood serum becomes turbid doubtless owing to the formation of numerous split products of protein breakdown. Such partially digested blood serum injected into normal skin induces prompt increased capillary permeability as well as marked leukocytic migration.

EXPERIMENTAL

Three types of experiments were set up as follows:

1. Serum was obtained from blood withdrawn by cardiac puncture from either rabbits or dogs.[1] A saline solution of trypsin (Merck)[2] was added to a test tube

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[1] Several experiments were likewise performed with horse serum.

[2] This material doubtless contains besides trypsin a number of extraneous substances.

containing an equal volume of blood serum. Three types of controls were set up. These consisted of serum and saline, of serum alone, and of trypsin and saline. The solutions were all incubated overnight at 37°C. A separate sample of untreated serum and one of the trypsin solution were kept in a refrigerator. On the following day the incubated solution of serum and enzyme appeared distinctly turbid; all the others were clear. About 0.4 cc. of each sample was injected intracutaneously into the dermis of the abdomen of a white rabbit. This was followed by the intravenous injection of 15 cc. of 1 per cent trypan blue in saline. The rapidity and intensity of local staining served as a rough measure of the rate of filtration through the endothelial wall. This test, as previously pointed out, is useful as an index of alteration in capillary permeability. About 40 minutes later, the cutaneous areas were excised and fixed in 10 per cent formaldehyde for microscopic study. The protocol of a type experiment is summarized in the following table. The number of plus signs indicates the intensity of local cutaneous staining.

Rabbit 16-83.

Cutaneous area inoculated with	Time of inoculation	15 cc. of 1 per cent trypan blue introduced at 10:50 — Presence of dye in inoculated area at			Migration of polymorphonuclear leukocytes Skin areas excised at 11:20
		10:51	10:54	11:10	
	hrs.:min.				
Rabbit serum + trypsin in saline; incubated	10 : 43	++	+++	++++	Extensive
Rabbit serum + saline; incubated	10 : 44	0	0	0	None
Rabbit serum; incubated	10 : 45	0	0	0	Few adhering polymorphonuclears to endothelial wall; but no outward migration
Trypsin in saline + saline; incubated	10 : 46	Trace at periphery	Trace to +	+	None
Serum; preserved on ice	10 : 47	0	0	0	"
Trypsin in saline; preserved on ice	10 : 48	Trace	Trace to +	Trace	"
Saline	10 : 49	0	0	0	None except for a rare and isolated cell in the perivascular tissue

The results of these observations indicate that the intracutaneous inoculation of the tryptic digest of blood serum induces a rapid in-

crease in capillary permeability subsequently followed by active leukocytic migration. The crystalline substance, leukotaxine, recovered from inflammatory exudates, manifests precisely similar biological properties (3, 4).

2. A specimen of blood serum was distributed into three containers. One part was treated with a solution of trypsin and incubated overnight at 37°C. Another was preserved in a refrigerator. The first and third sample were each fractionated with pyridine and acetone in accordance with the scheme previously described for the extraction and partial purification of leukotaxine (3). The final crystalline products contained probably a considerable admixture of sodium chloride inasmuch as further purification with butyl alcohol was omitted (3). The so called branched crystals, which are presumably sodium chloride associated with the active material, were abundantly present.

It is of interest to note that the tryptic digest of blood serum yields, on analysis, an acetone crystalline residue distinctly less soluble in water than that recovered from undigested serum. It is to be recalled in this connection that purified leukotaxine, extracted from an exudate, is relatively insoluble in water (3). The admixture of leukotaxine with sodium chloride yields a distinctly less soluble substance than the same salt, but essentially leukotaxine-free, recovered from normal serum (5). Furthermore, the crystalline fraction from the tryptic-treated serum was acid to phenol red in contradistinction to the alkaline reaction of the normal serum fraction. In brief, a comparison of the two types of end-products clearly indicates definite differences brought about by tryptic digestion of blood serum.

The results of an experiment are tabulated on page 156. The results indicate that the recovered crystalline acetone residue from the tryptic digest of blood serum is potent both in increasing capillary permeability and inducing migration of polymorphonuclear leukocytes. On the other hand, the crystalline solution derived from normal blood serum essentially fails to induce leukocytic migration. In the tryptic digest of blood serum, there is thus found a factor indistinguishable in its biological behavior from leukotaxine, in turn recovered from exudates (3). On the basis of these findings, the formation by enzymatic action of this active substance from normal blood serum does not seem an improbable inference.

Rabbit 16-88.

Cutaneous area inoculated with	Time of inoculation	15 cc. of 1 per cent trypan blue intravenously injected at 11:28 — Presence of dye in inoculated area at			Migration of polymorphonuclear leukocytes Skin areas excised at 12:12
		11:29	11:32	11:51	
	hrs.:min.				
Physiological saline	11 : 18	0	Tiny central speck	Tiny central speck	None
Crystalline residue of tryptic digest of blood serum; in saline	11 : 20	Trace	+ to ++	++ to +++	Considerable
Crystalline residue of untreated blood serum; in saline	11 : 26	0	Faint trace	Faint trace	Practically none
Blood serum	11 : 27	0	Trace at periphery	+ at periphery	" "

3. In an earlier preliminary note (6) we stated that a solution of crystalline serum albumin is unable *per se* to increase endothelial permeability. When, however, it is incubated with trypsin or urease, it acquires definite potency. Trypan blue accumulates readily from the circulating blood into cutaneous areas previously inoculated with the enzyme-treated solution of crystalline serum albumin.[3] At the time of these observations microscopic studies had been omitted. Several of these earlier observations have now been repeated with a few technical modifications.

A sample of crystalline trypsin was obtained through the courtesy of Dr. J. H. Northrop (7). This purified enzymatic preparation was added to an aqueous solution of crystalline serum albumin which had been previously freed of $SO_4^{=}$ by prolonged dialysis against distilled water. The mixture was incubated for a day at 37°C. and subsequently about 0.5 cc. was injected into the dermis of a rabbit. The results of an experiment are summarized in the following table.

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[3] This crystalline protein was obtained through the courtesy of Professor E. J. Cohn.

Rabbit 16-30.

Cutaneous area inoculated with	Time of inoculation	8 cc. of 1 per cent trypan blue intravenously injected at 10:02 Presence of dye in inoculated area at 10:09	10:15	10.28	Migration of polymorphonuclear leukocytes Cutaneous areas excised at 10·35
	hrs.:min.:sec				
Crystalline serum albumin in distilled water; incubated *for* 1 day	9 : 58 : 40	0 and blanching	0 and blanching	0 and blanching	None
Crystalline serum albumin in distilled water + crystalline trypsin; incubated for 1 day	9 : 59	+ at periphery	++ at periphery	++ to +++ at periphery	Adherence to endothelium and outward migration of cells
Crystalline trypsin in distilled water	10 : 00	Faint trace at periphery	Trace to + at periphery	Trace to + at periphery	None
Distilled water	10 : 00 : 15	0 and blanching	0 and blanching	Trace at periphery	"

The foregoing observations show that an inactive serum protein split by enzymatic hydrolysis yields a digest capable, upon cutaneous inoculation, of moderately increasing capillary permeability followed subsequently by migration of polymorphonuclear cells. This finding lends support to the hypothesis that leukotaxine, recovered from inflammatory exudates, is an intermediary product of protein catabolism (3). Previous studies indicated that leukotaxine probably belongs to the group of relatively simple polypeptides (2, 3). In the various experiments performed, such for instance, as the one presented above, on the incubation of crystalline trypsin with a solution of crystalline serum albumin, the end-products of the tryptic digest did not invariably induce leukocytic migration. The increase, however, in capillary permeability seemed to be a usual occurrence. It is therefore conceivable that the potency of the purified crystalline enzyme is such as sometimes to produce a high concentration of amino acids leaving behind a correspondingly small amount of non-

hydrolyzed polypeptides of the leukotaxine type. It is to be recalled in this connection that *alpha*-amino acids *per se* induce increased capillary permeability; but there is no available evidence that they likewise are accompanied by rapid leukocytic migration (8). Further studies are now in progress to clarify this question.

It is possible that the method herewith described may prove of practical significance. The conversion and extraction of leukotaxine from blood serum subjected to preliminary tryptic digestion may perhaps be a convenient method of obtaining an abundance of this active substance.

CONCLUSIONS

Blood serum digested by trypsin yields split products which, when injected into the skin of normal rabbits, rapidly increase the permeability of small cutaneous vessels. This is likewise followed in the affected tissue by prompt local migration of polymorphonuclear leukocytes.

The tryptic digest of blood serum can be purified by methods previously described (3). The resulting crystalline substance alters the permeability of the capillary wall and favors local migration of leukocytes in a manner similar to leukotaxine proper. The present observations indicate that leukotaxine, or a substance having at least identical biological properties, appears to have been formed by proteolytic enzymatic action on an otherwise relatively inactive blood serum. This finding serves as additional evidence in support of the view that leukotaxine, recovered from inflammatory exudates, is probably an intermediary product of protein catabolism.

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STUDIES ON THE RELATIONSHIP OF THE SEX HORMONES TO INFECTION

I. The Effect of the Estrogenic and Gonadotropic Hormones on Vaccinia and the Spreading Factor

By DOUGLAS H. SPRUNT, M.D., SARA McDEARMAN, and JAMES RAPER

(*From the Department of Pathology, Duke University School of Medicine, Durham, North Carolina*)

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In 1932 we reported (1) that pregnancy in rabbits altered the reactivity of the tissues to the virus of infectious myxomatosis, the secondary lesions being larger and the primary ones smaller in the pregnant animal than in the controls. As viruses are noted for their intimate parasitism it was thought that further study along this line would yield valuable information in regard to the host in the host-virus complex. Since pregnancy is a complex phenomenon we thought it desirable to try to obtain similar results by using only a few of the factors involved. It was thought that this could best be done by using some of the pregnancy hormones. In this paper we are reporting results which show that the estrogenic hormone, and perhaps also the gonadotropic hormone increase the resistance of the castrated rabbit to vaccinia and also that they inhibit the spread of India ink in the dermal tissues.

In the paper referred to above we reviewed much of the literature concerning the effect of pregnancy on the growth of tumors and resistance to infection. Many of these papers are contradictory and only a few of the most outstanding ones will be mentioned here. Bainbridge (2) in a summary of most of the work on tumors concludes that pregnancy stimulates the growth of most tumors with the exception of epitheliomas. Williams (3) in discussing the effect of pregnancy on infections concludes that with the exception of scarlet fever there is little to support the hypothesis that pregnancy increases the resistance of the body to infection. Since 1932 there have been several papers dealing with the effect of pregnancy or the sex hormones on infection. Pearce and her associates (4) showed in a large number of rabbits that pregnant animals were more resistant to vaccinia infection than non-pregnant ones and that females were more refractory than

males. Aycock (5) reported that the estrogenic hormone protected 5 out of 6 castrated monkeys against poliomyelitis. Kemp (6) reviews the rather extensive literature on the effect of the female sex hormones and pregnancy on the course of syphilis. He agrees with preceding workers that pregnancy modifies the course of syphilis, but was not able to confirm the work of others that this could be accomplished with estrogenic hormones.

In addition to this work on the effect of pregnancy on tumors and infection, there are a number of papers dealing with the production in various allergic states of changes associated either with pregnancy or resulting from the injection of the various sex hormones. Williamson (7) reports 13 patients who had less frequent attacks of asthma during pregnancy and 14 sensitive to pollen who showed no change during pregnancy. Manwaring (8) states that both pregnancy and parturition make it more difficult to sensitize a guinea pig.

Recently Solomonica and Kurzrok (9) reported that if anterior pituitary extract were given from 7 to 28 days before the guinea pigs were sensitized, it resulted in a modification of anaphylactic shock. This extract, if given after the animal was sensitized, had no effect in modifying anaphylactic shock. Amniotin, Squibb, when given after the animals were sensitized, caused a modification of anaphylactic shock. The effect of this preparation before the sensitizing period was not tried.

In an experimental study of anaphylactic shock in a large number of guinea pigs, we obtained results which suggested that the estrogenic hormone increased the susceptibility of the guinea pig to sensitization with a foreign protein, while the gonadotropic hormone made them more difficult to sensitize. There was, however, so much variation in the controls that a different method of approach seemed indicated. In addition to these papers Bray (10) cites a number of others which show a relationship between allergic manifestations and the female sex hormones.

Methods and Materials

Animals.—Young castrated adult male rabbits weighing about 2 kilos were used.

Operative Procedures.—The rabbits were castrated aseptically under ether anesthesia. This was done by making a small incision on both sides over the spermatic cord. The cord was freed and a suture placed around it. It was then cut and the testis and epididymis removed from the scrotal sac, after which the incision was sutured. The animals were kept for 3 weeks after the operation before the endocrine injections were started.

Endocrine Preparations.—The estrogenic preparation used was estrone and the gonadotropic preparation was anterior pituitary—like gonadotropic hormone of pregnancy urine.[1] The rabbits in the first experiment receiving the estrogenic

[1] Both of these were kindly supplied by Parke, Davis as theelin in oil and antuitrin S.

hormone were given 1000 international units per day for 24 days and the rabbits in the second experiment 2000 international units for the same period of time. The rabbits receiving the gonadotropic hormone were given daily injections of 400 rat units in the first experiment and 200 in the second experiment. All injections were given subcutaneously. The control animals were given 2 cc. of physiological saline daily.

Virus.—The virus used was the Levaditi strain of vaccinia obtained from Dr. T. M. Rivers. The material employed in all the experiments was a testicular suspension of this virus. In the first experiment only one injection of 0.25 cc. of a 10^{-4} dilution was given. In the second experiment 0.25 cc. of the following dilutions was injected intradermally 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8}. To minimize error the injections in all the animals were made from the same dilutions, the same syringe being employed for each.

India Ink.—This was used in a dilution of 1 part India ink to 2 parts Locke's solution. The injection of India ink was chosen for use in these experiments as we wished to compare our results with those obtained by Hoffman and Duran-Reynals (11). In the second experiment to avoid the possibility that repeated injections of the India ink in the same rabbit might affect the spread of subsequent injections only a few rabbits were used in the control period. It is interesting to note that this was an unnecessary precaution as no significant differences appeared in the control group between the animals which had had one injection and those which had had several injections. All the animals, however, showed a slight increase in the spread of India ink at the end of the experiment.

Measurement of Area.—The figures given in the various tables for the number of square millimeters covered by the vaccinia lesions and covered by the India ink are the product of their two diameters. More precise information might have been obtained by using a planimeter but it was found that this changed the results only slightly.

Wheal Disappearance Time.—This test, which consists in the injection of 0.2 cc. of physiological saline intradermally into the skin and noticing the time it takes for the disappearance of the wheal, has been used in studying certain intoxications. The literature concerning this test has been reviewed by Bradford (12). Although Hoffman and Duran-Reynals (11) report that the wheals produced by testicular extract disappear more quickly than those produced by saline, we have not found any studies giving exact data on the correlation of these two tests. A more detailed consideration of this factor will be made in a future paper. It is included in this paper merely in corroboration of the India ink experiment.

Necropsy.—At the conclusion of the experiment, all of the rabbits were necropsied and all the organs studied both in the gross and microscopically for any remaining testicular tissue and also for the presence of any extraneous disease. One rabbit was found which had received no endocrine injection but did have a caseous lesion of the lung. It is interesting to note that this animal which was excluded from the experiment resembled more the animals which had received the estrogenic hormone than it did the other control animals. Another rabbit from

which all the testicle had not been removed and which had received the estrogenic hormone showed a marked reduction in the spread of India ink and failed to show any lesions from the vaccinia. Two other rabbits died during the course of the experiment and were also excluded.

EXPERIMENTS

The experiments were designed to study the ability of the estrogenic and the gonadotropic hormones to modify the tissues of the host to virus infection and to ascertain the effect they had on the spread of India ink.

Experiment 1.—13 rabbits were used in this experiment. 5 were given the estrogenic hormone, 4 the gonadotropic hormone, and 4 were used as controls. They were vaccinated on one side and the India ink injected on the other. The vaccinia lesions showed no essential differences and hence are not shown. The area covered by the India ink after 1 hour is shown in Table I.

Experiment 2.—This experiment was designed to extend the findings of Experiment 1 in regard to both the spread of the India ink and the vaccinia infection. The objectives of the experiment were to determine whether the hormones actually decreased the spread of India ink or merely delayed it and to study the resistance to vaccinia infection by using several dilutions of the virus as described above.

24 rabbits were used in this experiment. 8 were given the estrogenic hormone, 8 the gonadotropic hormone, and 8 were kept as controls. The spread of the India ink was measured after 1 hour and again after 24 hours, the results being shown in Table II. It was thought that measurement of the spread from the moment of injection would be of interest. The amount of increased spread in 1 hour is given in Table III. The data on the disappearance time of the wheals and the effect on the vaccinia infection are given in Tables IV and V.

The rabbits were vaccinated, as described above, the day following the last injection of the hormones. The lesions were measured daily and the results tabulated in Table V. The rabbits were killed 14 days after vaccination.

Interpretation of Tables

Table I: The Spread of India Ink.—This table shows clearly that both the estrogenic and the gonadotropic hormones decrease the spread of India ink through the skin.

Table II: The Spread of India Ink.—This table shows that there is considerable variation in the spread of the India ink in the control animals. The variation, however, is about the same as that found by Hoffman and Duran-Reynals (11). If their figures are treated as ours were we find that in 11 controls the spread of the India ink

TABLE I

The Spread of India Ink

Rabbit No.	Estrogenic	Rabbit No.	Gonadotropic	Rabbit No.	Control
	sq. mm.		*sq. mm.*		*sq. mm.*
1	180	6	270	10	720
2	260	7	270	11	600
3	250	8	255	12	400
4	300	9	195	13	500
5	196				
Average....	237		247		555

TABLE II

The Spread of India Ink

Rabbit No.	Before injection		During injection Periods of time from first injection								14 days after stopping injections	
			7 days		14 days		21 days		23 days			
	1 hr.	24 hrs.	1 hr.	24 hrs.	1 hr.	24 hrs	1 hr.	24 hrs.	1 hr.	24 hrs.	1 hr.	24 hrs.
	sq. mm.	*sq. mm.*	*sq mm.*	*sq. mm*	*sq. mm.*	*sq. mm.*	*sq. mm.*	*sq. mm.*	*sq. mm.*	*sq. mm.*	*sq. mm.*	*sq. mm.*
					Estrogenic							
14	418	625	320	800	266	580	300	360	289	225	460	460
17	400	400	228	560	380	440	270	—	300	330	475	408
20					280	667	300	255	225	460	441	391
23					238	550	257	361	361	289	400	418
26							255	520	300	520	432	500
29							285	380	266	380	480	575
32							288	316	306	504	483	442
Average			274	680	291	559	279	365	293	358	453	477
					Gonadotropic							
15	418	400	320	525	304	—	340	460	532	768	460	460
18	420	352	340	494	374	374	475	1600	550	837	506	—
21			432	374	374	875	425	810	414	851	440	340
24					374	405	320	925	418	928	675	399
27							385	320	357	360	550	512
30							456	506	300	560	440	—
33							352	546	336	616	546	546
Average			364	464	356	551	393	738	415	703	517	451
					Controls							
16	440	400	352	475	418	456	440	440	238	400	456	460
19	342	400	396	500	380	304	360	650	462	440	504	375
22			300	500	336	330	500	—	414	594	484	520
25					320	—	351	500	396	640	425	399
28							440	616	396	660	450	475
31							385	500	400	560	462	728
Average	406	430	349	492	363	363	413	541	384	549	464	493

varied from 285 to 575 sq. mm. with an average spread 516 sq. mm. In the table there are 31 control readings which show a variation

TABLE III

The Increased Area Covered by India Ink after 1 Hour

Rabbit No.	Before injection	During injection Periods of time from first injection: 7 days	14 days	21 days	23 days	14 days after stopping injections
	sq. mm.	*sq. mm.*	*sq. mm.*	*sq. mm.*	*sq. mm.*	*sq. mm.*
			Estrogenic			
14	193	95	41	75	64	235
17	175	3	155	45	75	250
20			55	75	0	216
23			13	32	136	175
26				30	75	207
29				60	41	255
32				63	81	258
Average...		49	66	54	68	228
			Gonadotropic			
15	193	95	79	115	307	235
18	195	115	149	250	325	281
21		207	149	200	189	215
24			149	95	193	450
27				160	132	325
30				231	75	215
33				127	111	321
Average...		139	132	168	190	292
			Controls			
16	215	127	193	215	13	231
19	117	171	155	135	237	279
22		75	111	275	189	259
25			95	126	171	200
28				215	171	225
31				160	175	237
Average...	181	124	139	188	159	239

between 300 and 504 sq. mm. with an average of 413 sq. mm. Hoffman and Duran-Reynals (11) mention in their paper that they found no significant increase in the spread of the India ink after 1 hour. In

the table it will be observed that there is usually a slight increase in the spread of the India ink in the control animals but we do not believe that this is large enough to be considered significant.

TABLE IV

Wheal Disappearance Time

Rabbit No.	Before injection	During injection Periods of time from first injection				14 days after stopping injections
		7 days	14 days	21 days	23 days	
	min.	min.	min.	min.	min.	min.
			Estrogenic			
14	75	45	90	90	120	75
17	75		45	90	75	60
20			75	90	165	60
23			90	75	135	135
26				120	165	60
29				150	150	75
32				135	90	90
Average...			75	107	129	79
			Gonadotropic			
15	60	45	90	45	105	120
18	75		60	60	105	90
21			45	60	75	75
24			45	90	90	105
27				75	60	45
30				45	45	75
33				45	75	60
Average...			60	60	79	81
			Controls			
16	60	45	45	45	60	75
19	45		60	45	60	45
22			45	45	90	90
25			45	45	75	60
28				45	75	45
31				75	90	60
Average...	65	45	49	50	75	63

The animals which received the estrogenic hormone showed a decrease in the 1 hour spread of the India ink after the hormones had

been given for only 1 week. The spread of India ink after 24 hours was not only not decreased during the first 2 weeks but showed a

TABLE V

Vaccinia Lesions

Rabbit No.	Titration of virus	Maximum size of lesions 10^{-4}	10^{-5}	10^{-6}	10^{-7}	Maximum size of lesions at end of experiment
		sq. mm.	*sq. mm.*	*sq. mm.*	*sq. mm.*	*sq. mm.*
		Estrogenic				
14	100×10^{-7}	320	49	0	0	0
17	100×10^{-7}	100	36	0	0	4
20	1000×10^{-7}	240	0	0	0	9
23	100×10^{-7}	400	256	0	0	121
26	100×10^{-7}	400	144	0	0	36
29	100×10^{-7}	360	324	0	0	165
32	100×10^{-7}	625	225	0	0	150
Average...	229×10^{-7}	349	172			69
		Gonadotropic				
15	10×10^{-7}	270	180	121	0	25
18	10×10^{-7}	352	272	288	0	100
21	10×10^{-7}	360	121	81	0	100
24	10×10^{-7}	225	400	225	0	144
27	100×10^{-7}	594	225	0	0	550
30	1×10^{-7}	400	144	25	9	36
33	1×10^{-7}	324	81	0	81	64
Average...	20×10^{-7}	361	203	106	13	145
		Control				
16	10×10^{-7}	550	196	9	0	120
19	1×10^{-7}	625	144	25	4	625
22	10×10^{-7}	256	84	100	0	81
25	1×10^{-7}	500	144	144	144	500
28	100×10^{-7}	340	100	0	0	16
31	100×10^{-7}	308	100	0	0	0
Average...	37×10^{-7}	430	128	46	25	224

slight increase over the controls. After 3 weeks the spread of India ink was decreased after 24 hours as well as 1 hour.

It should be observed in Table II that in the 1 hour readings of 31

controls there is only 1 instance below 300 sq. mm., whereas the 20 one hour readings while the estrogenic hormone was being given show only 4 instances above 300. The same applies to the 24 hour readings of those which had received the estrogenic hormones for 3 weeks, there being only 3 above 400 sq. mm. in this group and in the control group only 4 below 400 sq. mm.

It will be seen both in Table II and Table III that the gonadotropic hormone did not cause a decrease in the spread of India ink as it did in Experiment 1.

Table III: The Increased Area Covered by India Ink after 1 Hour.—This table shows the increase in size of the area covered after 1 hour rather than the total area. This is done to bring out more clearly the effect of the estrogenic hormone in limiting the spread of the ink.

Table IV: Wheal Disappearance Time.—The results of this experiment run parallel to the India ink one in that the longer the wheal persisted the less was the spread of the India ink. The disappearance time of the wheals had returned to normal in every case 2 weeks after discontinuing the injection of the hormones.

Table V: Vaccinia Lesions.—This table shows that the rabbit's resistance to infection with vaccinia is increased if the rabbit has been given large amounts of the estrogenic hormone before it is vaccinated. There does not, however, appear to be any great difference in the maximum size of the lesion if the virus was able to take hold. It also shows that if the virus is of sufficient strength to cause a lesion in the animal then this lesion will in most instances reach the same size as the ones in the control group. This is in agreement with Experiment 1. The average size of the lesions at the end of the experiment shows that the animals given the estrogenic hormone recovered from their skin lesions more quickly than the control animals. However, since there is such wide variation in size we prefer to wait until we have further data before stressing this point.

DISCUSSION

At present it does not seem advisable to attempt to explain how these changes occur except that they apparently are related to the permeability of the tissue to both fluids and particulate matter. It does appear, however, that there is present in the estrogenic hormone

some factor or factors that have an effect on the tissues of the body opposite to that of simple extracts of the testis. It is thought that further study of this factor or factors will lead to a better understanding of the modifications which have been seen to occur as a result of pregnancy in tumor growth, infection, and allergic states. It also gives us a new method of studying the reactions of the host to numerous incitants of disease.

CONCLUSIONS

The essential findings of these experiments may be summarized as follows:

The estrogenic hormone after being given for 1 week slows up the spread of India ink but allows it to reach and exceed a normal spread after 24 hours.

After injections of the estrogenic hormone for 3 weeks the spread of India ink is much less than in the control animals.

The resistance of the rabbit to vaccinia is increased if the rabbit has been castrated and then given the estrogenic hormone for a period of 3 weeks before being vaccinated.

At the present time nothing can be said about the action of the gonadotropic hormone on India ink, as the experiments did not agree.

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ANAPHYLAXIS IN THE ISOLATED HEART

By HERBERT B. WILCOX, Jr.,* M.D., and E. COWLES ANDRUS, M.D.

(From the Cardiographic Laboratory, Department of Medicine, Johns Hopkins University and Hospital, Baltimore)

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Evidence is not wanting that the heart is affected in the process of anaphylactic shock. Morphological alterations following the injection of foreign protein into sensitized animals, local areas of degeneration of the myocardial cells with round cell infiltration or, later, scar formation, have been reported by Longcope (11), Klinge (9), and by Seegal, Seegal and Jost (14). Electrocardiograms recorded in the intact animal during anaphylactic shock, demonstrating disturbances of conduction and abnormalities of origin and spread of the excitatory process in the heart, have been described by Auer and Robinson (1), Hecht and Wengraf (8), Koenigsfeld and Oppenheimer (10) and by Criep (5). But the exact nature of these effects, whether intrinsic, or secondary to asphyxia (5), or to the remote elaboration of substances which secondarily react upon the myocardium (3), is not clearly defined.

The proof that any organ is intrinsically affected by the process of anaphylaxis must rest upon certain distinct criteria: (*a*) It should be shown that the capacity of the tissue to react specifically upon exposure to the appropriate antigen survives its isolation from the remainder of the animal and persists in the organ perfused or immersed in artificial solution, and, (*b*) the particular reaction should be evoked by doses of antigen which fail to affect an organ isolated from an unsensitized or desensitized animal and should diminish in intensity or disappear with successive exposures thereto.

The essential features of anaphylaxis have, indeed, been demonstrated in this fashion *in vitro* upon isolated segments of intestine (6) from sensitized guinea pigs and upon the isolated uterus (13) from similar animals. Reactions which fulfill the above criteria have been observed to occur in the isolated hearts of warm blooded animals by Caesaris-Demel (4), Mendeleeff, Hannevart and Platounoff

* Jacques Loeb Fellow in Medicine, Johns Hopkins University.

(12), and by Went and Lissák (18). The latter report that, as recorded by a myographic tracing, the heart beat is depressed, sometimes after preliminary acceleration, following the introduction of small quantities of antigen into the perfusing fluid.

The observations reported herein were undertaken with a view to analyzing the anaphylactic reaction by means of simultaneous myographic and electrocardiographic records, together with measurements of the rate of flow of the perfusate through the isolated heart.

Method

Young guinea pigs were rendered sensitive to horse serum by the intraperitoneal injection of 0.1 to 0.5 cc. 3 to 5 weeks later the hearts of these animals were isolated and perfused with Ringer-Locke solution at 35°C. and a pressure of 75 mm. Hg by a modification of the Langendorff technique. A myographic tracing of the ventricular beat was recorded by means of a thread attached to the right ventricle. Electrocardiographic records were obtained by contact electrodes of worsted wet with salt solution attached to tissue of the superior mediastinum removed with the heart and to the apex of the left ventricle.

One detail of this technique deserves particular reference. In this preparation, the inflow cannula being tied into the aorta, the perfusate may follow one of two courses. By far the major portion thereof enters the coronary arteries in the sinuses of Valsalva, flows through the coronary vessels, and leaves them largely through the coronary sinus into the right auricle or *via* those Thebesian channels which empty into the right heart. In the present experiments this fluid has been collected by means of a siphon cannula thrust into the right ventricle through the pulmonary artery, the venae cavae having been previously ligated, and has been measured by means of an electric drop recorder. This, though not actually the entire coronary flow, but certainly a large aliquot thereof, will be referred to below as C flow. The remainder of the perfusate passing through the coronary circuit escapes through those Thebesian vessels which empty into the left heart. This, plus such fluid as may regurgitate past the aortic and mitral valves, finally escapes through the severed pulmonary veins or through a small hole made into the cavity of the left ventricle. Constituting about one-fourth of the total flow this will hereinafter be called leakage flow. Since, when recorded, this leakage flow has always been found to vary in the same direction as the C flow it has not been registered in all the experiments.

The antigen diluted to 1 cc. with warm Ringer-Locke's solution was introduced by injection through the wall of the rubber tubing into the stream of the perfusing fluid immediately above the cannula.

RESULTS

The injection of 0.01 to 0.1 cc. of horse serum into the perfusate about to enter the heart removed from a normal guinea pig, so isolated,

is without significant effect. In a small proportion of instances the larger doses may provoke a slight evanescent change in the amplitude of contraction or an occasional extrasystole. It is noteworthy that such effects, when observed, are completed within 8 to 20 seconds after the injection and are unaccompanied by demonstrable changes in C or leakage flow. Identically negative results are obtained when horse serum in such amounts is brought into contact with a heart removed from an animal after fatal anaphylactic shock evoked *in vivo* by the same antigen, or with a heart previously sensitive *in vitro* but desensitized by one or more exposures to small amounts of antigen in the perfusate.

When, on the other hand, 0.002 to 0.005 cc. of serum is introduced into the heart removed from a guinea pig previously sensitized with the same, there ensues an entirely different series of events. There is no noticeable effect for about 1 minute. Then occur a striking acceleration of the heart rate and alteration in the amplitude of contraction. Electrocardiograms taken at intervals before and after the onset of this reaction disclose prolongation of the P–R interval, changes in form of the QRS and T complexes, and ectopic rhythms. In a number of instances the ventricular rate has exceeded the auricular, producing auriculoventricular dissociation with ventricular tachycardia. All these phenomena pass off within 5 to 15 minutes and the myographic and electrocardiographic records reassume the form present before the injection of serum. Subsequent injections of even larger amounts of serum produce less pronounced reactions or none.

The magnitude of the cardiac acceleration observed in eight such experiments with the chief alterations in the electrocardiographic records in each instance are summarized in Table I. Portions of representative electrocardiograms are illustrated in Text-fig. 1.

But in the present study an additional effect, which is presumably of primary importance, has been observed invariably to accompany the phenomena of cardiac anaphylaxis just described. In every instance the reaction has involved a striking reduction in the rate of coronary (C) flow. This is illustrated in Text-fig. 2, in which are depicted cardiac rate and coronary (C) flow in a characteristic experiment, and in Table II*a*. In many instances the sudden drop in

coronary flow has been observed to precede, by a perceptible interval, the typical myographic and electrocardiographic changes. That these variations in the rate of C flow represent actual changes in caliber of the coronary vascular system, and are not due simply to increase in heart rate or tonus, is demonstrated by such experiments as those illustrated by Text-fig. 3 and Table III, wherein the heart was driven at a constant rapid rate by means of induction shocks. The characteristic reduction of C flow following the administration of antigen still takes place when the cardiac rate is controlled. Moreover, the coronary flow is not slowed by the introduction of antigen in such

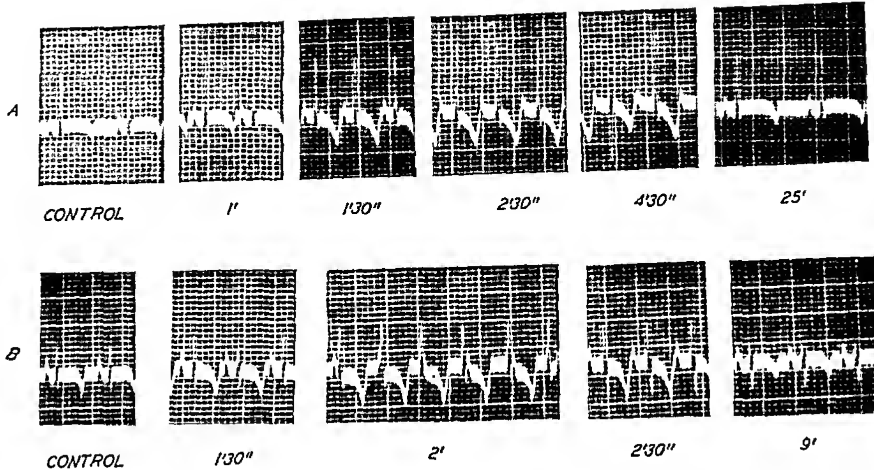
TABLE I

Date	Initial rate (beats per min.)	Maximum rate after serum (beats per min.)	Interval between injection of serum and maximum effect	Electrocardiographic alterations
			min. : sec.	
Nov. 16	144	215	1	A-V dissociation with nodal tachycardia
" 30	125	200	1 : 40	Ventricular extrasystoles. Ventricular tachycardia
Dec. 2	135	220	1 : 30	Sinus tachycardia. Prolonged P-R interval
" 3	210	250	1 : 10	Sinus tachycardia
" 14	178	210	1 :	" "
" 17	155	220	1 : 30	" "
" 21	180	260	1 : 30	A-V dissociation. Ventricular tachycardia
Jan. 6	170	220	2	" " " "

These are samples of 32 similar reactions observed.

an amount into the driven normal heart or following desensitization of a previously sensitive organ. The magnitude of this effect and the regularity with which it is produced suggests that it is fundamental to the anaphylactic reaction of the heart in this species. Similar results have been observed in a small number of experiments with the hearts of sensitized rabbits. In both species the electrocardiographic changes are strikingly similar to those recorded after mechanical constriction of the coronary arteries.

The effect of the interaction of antigen and antibody upon the cardiac tissue devoid of coronary circulation is not, of course, to be discerned in the results of such experiments, but Baker (2) has demon



TEXT-FIG. 1A. Sample electrocardiograms show progressive increase in the P–R interval from 0.07 seconds, reaching a maximum of 0.12 at $4\frac{1}{2}$ minutes after introduction of antigen, where an extrasystole of supraventricular origin also appears, returning to the initial value at 25 minutes. Transient increase in rate and in the depth of the T waves is also illustrated, together with depression of the S–T segment.

TEXT-FIG. 1B. Electrocardiograms of another experiment showing similar changes in T waves and S–T segments; and, at $1\frac{1}{2}$ minutes, alternate excitations arising in two separate supraventricular foci, one with a P–R interval of 0.07 seconds and the other 0.09 seconds; at 2 minutes auriculoventricular dissociation with the ventricular rate slightly more rapid than the auricular.

TABLE II*a*

Date	Cardiac rate (beats per min.)			C flow (drops per min.)			Perfusate
	Before serum	After serum	Increase	Before serum	After serum	De-crease	
			per cent			*per cent*	
Feb. 1(1)	150	225	50	97	30	69	Ringer-Locke
" 1(2)	160	240	50	100	30	70	" "
" 4	211	250	19	100	32	68	" "
Mar. 12	160	210	31	110	40	63	" "
" 29	120	180	50	110	14	87	" "
July 13	162	198	22	96	42	56	" "
" 14	150	216	44	114	54	53	" "
June 2*	180	202	12	186	66	66	" "
Average........			34.7			66	

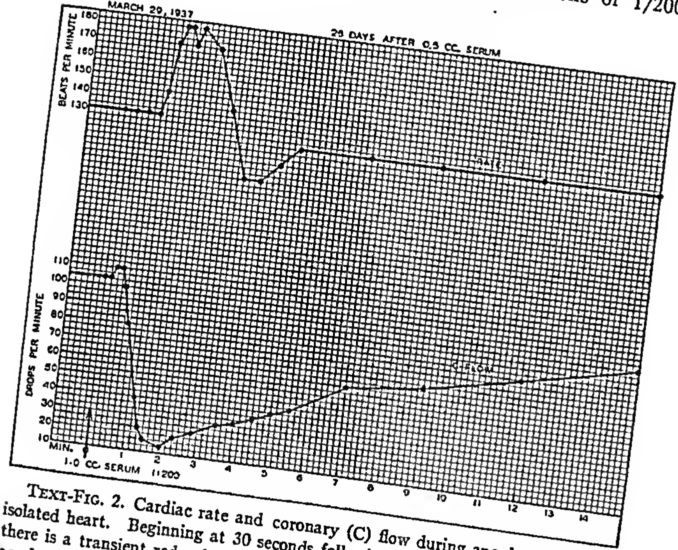
TABLE II*b*

Date	Cardiac rate (beats per min.)			C flow (drops per min.)			Perfusate
	Before serum	After serum	Increase	Before serum	After serum	Decrease	
			per cent			*per cent*	
July 7(1)	168	228	36	168	144	14	Atropine 1/100,000 in Ringer-Locke
" 7(2)	204	228	12	198	186	6	" " " " "
" 1*	162	204	25	120	120	0	" " " " "
May 10	170	220	29	180	120	33	" " " " "
" 27	198	252	27	102	66	35	" " " " "
" 26	180	240	33	54	33	39	" " " " "
July 8(1)	204	246	21	108	108	0	" 1/75,000 " " "
" 8(2)	150	222	48	156	102	35	" " " " "
" 12	156	198	26	162	162	0	" 1/50,000 " " "
" 13	168	210	25	102	72	30	" " " " "
Average...........			27.5			21.6	

* Passive sensitization.

strated depression of the activity of the auricles of sensitized rabbits upon exposure to minute amounts of antigen and Sherwood (15) has described anaphylactic effects in the hearts of chick embryos

2 to 7 days old, before any extensive coronary circulation has developed. On the other hand, Wachstein (17) has reported that heart muscle strips from normal and from sensitized rabbits and guinea pigs react similarly to horse serum in dilutions of 1/200 to 1/2000.

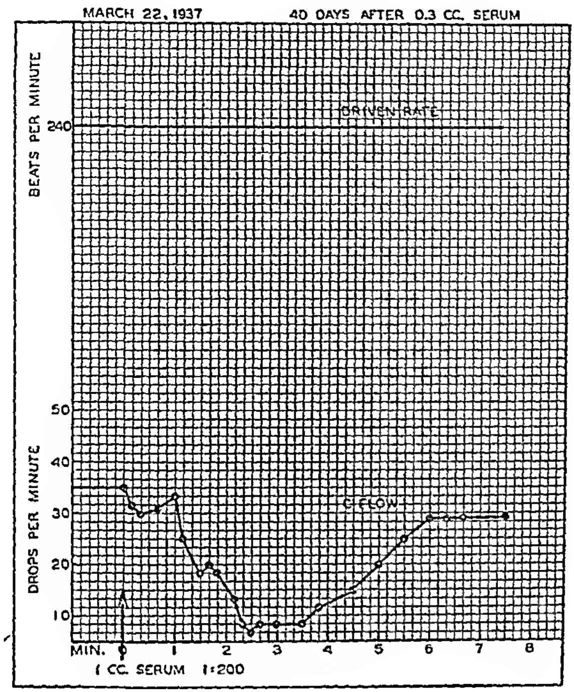


TEXT-FIG. 2. Cardiac rate and coronary (C) flow during anaphylaxis in the isolated heart. Beginning at 30 seconds following the introduction of antigen there is a transient reduction in coronary (C) flow which slightly precedes the acceleration in the rate of contraction.

In this and other charts each vertical division represents an interval of 10 seconds.

In view of the current hypothesis (7) that the manifestations of anaphylaxis are consequent upon the elaboration of histamine or an histamine-like substance, the effects of histamine have been tested upon this same preparation. Text-fig. 4 illustrates that certain transient changes identical with those observed in cardiac anaphylaxis are produced when a small amount of histamine is introduced

into the perfusate about to enter the heart: increase in rate of beat, transient auriculoventricular dissociation and a striking reduction



TEXT-FIG. 3. Anaphylactic reaction with ventricular rate controlled.

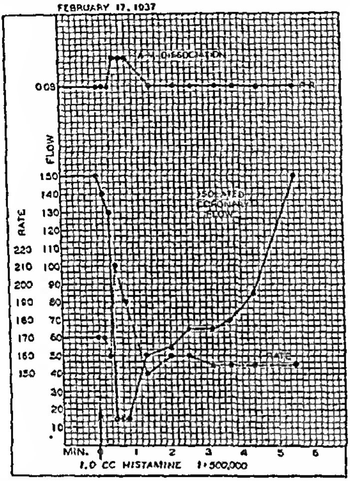
TABLE III

Date	Driven rate (beats per min.)	C flow (drops per min.)		
		Before serum	After serum	Change
				per cent
May 22	240	35	7	80
Mar. 24	210	85	50	41
" 25	320	78	21	73
" 30	350	45	20	55

Note that different driven rates were employed. 4 such experiments done.

in C flow. This latter is demonstrable though the ventricular rate be controlled by driving. Several experiments are summarized in Table IV*a*. In contrast, however, with the effect of serum, the

action of histamine upon the isolated preparation is the same whether the heart be that of a normal or a sensitized animal, or one which has been desensitized by previous exposure to antigen. Moreover, the reaction to histamine itself invokes no demonstrable degree of desensitization; subsequent exposure to the same dose of histamine, or, in the case of sensitive hearts, to a small amount of antigen, is followed by a typical reaction undiminished in intensity.



TEXT-FIG. 4. Cardiac rate and coronary (C) flow as influenced by histamine.

Finally, a series of observations was made to study the effect of atropine upon the phenomena of anaphylaxis in the heart and to compare this with the influence of atropine upon the reaction to histamine. The results are summarized in Tables II and IV.

With hearts which are demonstrably sensitive to horse serum the presence of atropine in concentrations of 1/100,000 to 1/50,000 in the perfusate has little or no effect in preventing the acceleration of cardiac rate typical of the anaphylactic reaction. Upon the changes in coronary (C) flow the effect of atropine is distinct; in all of the ten

experiments recorded in Table II*b* the reduction of coronary flow is considerably less than in those of Table II*a* in which the perfusate

TABLE IV*a*

Ringer-Locke Perfusate

Date	Dose of histamine	Cardiac rate (beats per min.) Before	After	Change	C flow (drops per min.) Before	After	Change	Remarks
				per cent			*per cent*	
Feb. 17	0.5 cc. 1/500,000	150	210	40	150	30	80	Normal pig
" 18	" " "	180	190	5.5	170	80	53	" "
" 24	" " "	130	220	69	80	25	62	Desensitized *in vivo*
Mar. 30*	" " "	300	300		45	16	64	Desensitized *in vitro*
Apr. 22	" " "	160	180	12.5	95	38	60	" " "

These are samples from a group comprising 15 animals and 26 injections; in 4 such observations the contraction rate was controlled by driving.

TABLE IV*b*

Atropine 1/100,000 in Ringer-Locke Perfusate

Date	Dose of histamine	Cardiac rate (beats per min.) Before	After	Change	C flow (drops per min.) Before	After	Change	Remarks
				per cent			*per cent*	
Feb. 17	1 cc. 1/500,000	130	150	15	102	102	0	Normal
Apr. 19	0.5 " "	170	230	35	100	100	0	"
May 12	" " "	144	216	50	150	144	<1	"
July 7	" " "	180	210	16	108	108	0	Desensitized *in vitro*
Mar. 17	" " "	170	190	12	85	85	0	Normal
Apr. 22	" " "	150	165	10	120	120	0	Desensitized *in vivo*

These are samples from 17 injections on 12 animals.

* Driven.

contained no atropine. Indeed, in three instances, although the cardiac rate increased during "shock" by 20 to 25 per cent, the coronary flow changed not at all.

The effect of histamine in constricting the coronary arteries in this species is almost completely suppressed by atropine in the above concentrations though its cardio-accelerator action is little influenced thereby (see Table IV*b*). This effect is in confirmation of that reported by Viotti (16). Here again identical results are obtained whether the effect is produced in the hearts of normal animals or in those desensitized *in vivo* or *in vitro*.

In the sense that the effect of atropine upon the reactions of the guinea pig's heart to both anaphylaxis and to histamine are *qualitatively* similar, these observations lend support to the view that the manifestations of anaphylaxis are due to the elaboration of histamine or an histamine-like substance. In that the phenomena characteristic of anaphylaxis are here *quantitatively* less susceptible of inhibition by atropine than are the effects of histamine, it may be urged that, in the anaphylactic reaction, additional substances may be involved.

Went and Lissák (19) have suggested that the active material elaborated during anaphylactic shock may be choline rather than histamine; in their experiments anaphylaxis was characterized by depression rather than stimulation of the heart beat. The results reported herewith indicate that, if substances other than histamine are produced in this reaction, such substances are atropine resistant. In either case it seems plain that the heart of a sensitized animal may be profoundly influenced by the reaction of antigen and antibody taking place in its own tissue aside from any secondary effect of substances elaborated elsewhere in the body, and that one fundamental characteristic of this anaphylactic reaction in the guinea pig is a conspicuous constriction of the coronary arteries.

SUMMARY

The isolated hearts of guinea pigs sensitized to horse serum have been shown to react characteristically upon exposure to small amounts of antigen. The cardiac rate is temporarily accelerated and transient alterations in amplitude of contraction are to be observed. Electrocardiographic abnormalities, previously recorded by remote leads during anaphylactic shock in the intact animal, have been recorded by direct leads from the isolated perfused hearts of sensitized animals during this reaction.

An additional effect of anaphylaxis in the isolated heart of the guinea pig is reported: a striking reduction in the rate of flow through the coronary vessels.

The anaphylactic reaction of the isolated heart of the guinea pig has been compared with the action of histamine upon the same preparation and the effect of atropine upon each has been observed. The implications of certain quantitative differences in the influence of atropine upon these reactions are discussed.

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QUANTITATIVE STUDIES ON ANTIBODY PURIFICATION

II. The Dissociation of Antibody from Pneumococcus Specific Precipitates and Specifically Agglutinated Pneumococci*

By MICHAEL HEIDELBERGER, Ph.D., and ELVIN A. KABAT, Ph.D.

(From the Department of Medicine, College of Physicians and Surgeons, Columbia University, and the Presbyterian Hospital, New York)

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In the first paper of this series (1), it was shown that quantitative study of the effect of salts on the precipitin reaction between pneumococcus polysaccharide and homologous antibody (2) had provided a theoretical basis for the dissociation of pneumococcus specific precipitates with strong salt solutions, since, in these solutions, the polysaccharide combined with less antibody than at physiological concentrations. As a result, it was found possible to pass in a single step from unconcentrated Types I and II antipneumococcus horse sera and Type III antipneumococcus rabbit sera to antibody solutions of which 85 to 93 per cent of the total nitrogen was immune nitrogen.

Less satisfactory results were obtained with Type III antipneumococcus horse sera, but in the present studies it is shown that this difficulty can readily be overcome. The application of the dissociation procedure to precipitates formed by the specific polysaccharide of Type I pneumococcus and Type I antipneumococcus rabbit sera gave irregular results, and attempts at the analysis of these irregularities are recorded. Experiments on the dissociation of Type VIII and C-anti-C precipitates are described and preliminary data are given on the extension of the dissociation procedure to specific precipitates formed by pneumococcus polysaccharides in antisera from pigs and sheep, and in a bovine antiserum. The last, a low grade

* The work reported in this communication was carried out under the Harkness Research Fund of the Presbyterian Hospital.

serum, from an animal injected with Types I, II, and III pneumococci, readily yielded antibody to each in a high state of purity, the Type III antibody being analytically pure, or 100 per cent specifically precipitable.

For the formation and dissociation of pneumococcus specific precipitates it is necessary to isolate the specific polysaccharides, although it is probable that relatively crude preparations would suffice. Since there is now ample evidence that specific precipitation and specific bacterial agglutination are manifestations of the same chemical reactions (for a discussion, see (3)), it seemed probable that the salt dissociation procedure could be applied more simply to agglutinated pneumococci. It had been shown that the effect of salts on the agglutination of pneumococci by specific antiserum (3) is similar to their effect on specific precipitation, and the possibility of dissociating antibody from agglutinated pneumococci had already been demonstrated by Chickering (4) and Huntoon (5*a*), and quite recently by Chow and Wu (6). Salt dissociation of antibody from agglutinated *Salmonella* has also been studied by Duncan (5*b*). It is shown below that salt dissociation of specifically agglutinated pneumococci is readily effected, and that in some instances both the yield and degree of purity of the resulting antibody are higher than of the antibody from specific precipitates formed in the same sera. Moreover, the barium hydroxide modification (1) of Felton's alkali dissociation procedure (7) is also applicable to the residual undissociated agglutination complex.

Finally, a discussion is given of the significance of the findings and of several questions raised by recent work of Chow and Wu (6).

EXPERIMENTAL

1. Materials and Methods.—The specific polysaccharides used were prepared by the relatively mild procedures described in (8). Pneumococcus Type I suspensions consisted either of heat-killed organisms, or formalinized and heated suspensions as recommended recently (9) on account of their stability. Before use all suspensions were washed with saline until the washings were practically free from specific polysaccharide[1] in order to avoid the occurrence of precipitin reactions with the agglutination reaction it was desired to study. The horse sera used were

[1] Referred to throughout as S, with the appropriate numeral to designate type.

obtained from the New York City Department of Health through the courtesy of Dr. Wm. H. Park, Dr. K. G. Falk, and Miss A. W. Walter.

Analyses for precipitin nitrogen were made according to (10–12) by addition of a slight excess of homologous S to duplicate 1 to 5 ml. portions of the chilled antibody solution and determination of the nitrogen in the washed precipitate after 48 hours in the cold. Since the total nitrogen in the supernatants was extremely low, one washing with 2 to 3 ml. of chilled saline was considered sufficient. Blank tubes were run similarly with antibody solution alone, and micro Kjeldahl estimations were run separately on the supernatants (plus washings) of the blank tubes and on the usually negligible residues, the sum of the two giving the total nitrogen of the antibody solution. In the analyses of Type I antibody the small amount of S I nitrogen precipitated was deducted from the total nitrogen precipitated in order to give antibody nitrogen. As large aliquot portions as possible of the supernatants from the precipitin determinations were analyzed for agglutinin nitrogen according to (13), by addition to a measured volume of a suspension of homologous type specific pneumococci (Pn) and estimation of the increase in nitrogen over that in the Pn suspension alone after centrifugation and a single washing.[2] In some instances the total antibody nitrogen was determined by the agglutination method. For convenience analyses were calculated to the somewhat uncertain third decimal place.

2. Dissociation of Antibody from Specific Precipitates Formed in Antipneumococcus Sera of Various Species.—15 to 860 ml. of type specific antipneumococcus serum, or, in one instance Felton antibody solution (14), were precipitated at 0°C. unless otherwise indicated in the tables, with an amount of homologous S calculated to bring the system to the beginning of the equivalence zone (15) or to leave a small excess of antibody. After the precipitate had flocked, the mixture was decanted or centrifuged in the refrigerated centrifuge and the precipitate was evenly suspended in chilled saline and washed repeatedly with this until the amount of heat-coagulable protein extracted was at a minimum. Usually 4 to 7 washings were sufficient. Precipitates from Type I antipneumococcus rabbit sera obtained by successive small additions of S I, following a suggestion of Chow and Wu's (6), appeared more difficult to wash than those in which the S I had been added in a single portion. In the latter instance, however, more S was required, and the resulting antibody solution was not necessarily better than when the former procedure was used.

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[2] In applying differential analysis of supernatants for agglutinins (1) to whole sera, Goodner, Horsfall, and Dubos (9) found it necessary to use such small aliquots that the final result was multiplied by ten to give agglutinin in mg. per cc. The errors of three separate analyses are thus multiplied tenfold, and when these are cumulative, the product could easily equal the entire antibody content of low grade sera. It is to be regretted that Table 6 in the paper referred to does not include comparisons with the standard method (13). The necessity for ensuring an excess of Pn is also not stressed.

Many of the precipitates formed and washed at 0°C. were given one or two subsequent extractions with saline at 37°C. until the amount of protein removed was again at a minimum. These extracts were concentrated by dialysis against saline under negative pressure and analyzed for their antibody content (see tables).

After the saline washings the precipitates were extracted at about 37° with 10 to 40 ml. of 15 per cent sodium chloride solution (*cf.* 1) for 1 hour in the presence of a drop of toluene, and after centrifugation were usually extracted again with a smaller volume of the salt solution. In many instances the residual precipitates were suspended in water and treated in the cold with 0.5 to 1 ml. more of saturated barium hydroxide solution than necessary to dissolve the precipitate. After 1 hour in the cold 1 to 4 ml. of 10 per cent barium chloride solution were added and the mixture was neutralized with dilute acetic acid and centrifuged. The supernatant usually contained much antibody (*cf.* 1). Some of the precipitates from rabbit sera were very difficult to dissolve with barium hydroxide unless previously washed several times with water to remove salt and allowed to swell in water. All solutions were dialyzed in the cold against 0.9 per cent saline in the presence of a little toluene and were concentrated at the same time under negative pressure. Dialysis was continued until interferometer readings showed no increased salt concentration in the outer liquid; or, in the case of the barium hydroxide-treated solutions, until the outside saline was free from barium ion.

In using 15 per cent salt solution for the dissociation it was usually noted that a solubility effect was superimposed upon the equilibrium-shifting effect which occurred almost exclusively when 10 per cent sodium chloride solutions were used (1, 2). In the case of the stronger solutions precipitation usually occurred before dialysis had advanced very far. The precipitates were preferably centrifuged off in the cold before continuing the dialysis in order to prevent their reacting with additional antibody as the salt content diminished.

In Table I are given data on antibody recovered from Types III and VIII antipneumococcus horse sera. Since absorption with pneumococcus protein, as in preparation 792 L, seemed of no advantage, other lots of these sera were absorbed only with pneumococcus C substance (16) before precipitation with S. The same low grade Type III serum was used as in (1), so that the higher purity of the antibody seems due to the more thorough washing given the specific precipitate before dissociation. The Type VIII sera were precipitated with S III in order to study differences in the cross reactivity of the dissociated antibody from that of the original serum.

In Table II are given data on the dissociation of C-anti-C precipitates obtained in the preliminary absorptions of the three anti-Pn horse sera used for the experiments recorded in Table I. The C precipitates were washed in the cold as usual. The seventh and final washing of the precipitate from serum 644 still contained considerable protein and antibody. The residues were then extracted several times with saline at 37°C. (solutions C_1 and CC_1) and finally with 15 per cent salt solution at 37°C. (solutions C_2 and CC_2). Recovery of anti-C was very poor, but the 37° saline extracts contained surprisingly large amounts of type specific anti-

body. Attempts to recover anti-C from precipitates in Type I antipneumococcus rabbit sera were even less encouraging, nor could anti-S I be recovered at 37° from the C-anti-C precipitates previously washed at 0°.

TABLE I

Antibody Solutions from the Dissociation of Specific Precipitates from Types III and VIII Antipneumococcus Horse Sera

Serum and preparation number	Antibody N taken	Precipitin + agglutinin N in recovered antibody solution	Recovery of antibody N	Total N	Precipitin N	Agglutinin N	Antibody N	Antibody N / Total N: 37°, 0.9 per cent NaCl extract	Antibody N / Total N: 15 per cent NaCl extract	Antibody N / Total N: $Ba(OH)_2$ dissociated solution
				per ml. of antibody solution						
	mg.	*mg.*	*per cent*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*	*per cent*
Type III										
792 L	70.5	16.8	24	0.322	0.282	0.015	0.297		92	
N*	39.5	9.1	23	0.251	0.230	0.000	0.230		92	
P†	28.2	6.4	23	0.183	0.167	0.000	0.167		91	
Q	67.7‡	11.2(P)	17	0.322	0.295		0.295(P)			92(P)
{R	41.4	1.1	3	0.074	0.027	0.013	0.040	54		
{T		9.9	24	0.236	0.223	0.009	0.232		98	
{DD	196	1.6	0.8	0.271		0.101	0.101	37		
{EE		24.3	12	0.908	0.860	0.008	0.868		96	
{FF		4.5	2.3	0.310	0.154	0.029	0.183			59
Type VIII; serum precipitated with S III										
644 {D	77.0§	3.1(P)	4	0.194	0.123§		0.123(P)	63(P)		
				0.194	0.169‖		0.169(P)	87(P)		
{E		13.6(P)	18	0.697	0.665§		0.665(P)		95(P)	
				0.697	0.686‖		0.686(P)		98(P)	
909 {D	53.8§	3.4(P)	6	0.210	0.170§		0.170(P)	81(P)		
				0.210	0.195‖		0.195(P)	93(P)		
{E		8.5(P)	16	0.373	0.345§		0.345(P)		92(P)	
				0.373	0.356‖		0.356(P)		95(P)	

(P) after analyses indicates precipitin N only.

* Serum precipitated first with 0.2 mg. S III to remove much of precipitable lipids; precipitate discarded.

† Precipitated at 37°, washed with chilled saline.

‡ Residues, after N and P had been extracted, dissociated by $Ba(OH)_2$ method (1).

§ With S III.

‖ With S VIII.

Experiments on the dissociation of specific precipitates from Type I antipneumococcus rabbit sera are summarized in Table III. Solution 193 C was obtained by 15 per cent salt dissociation of the precipitate from S I and two pooled sera of rather low antibody content, without previous absorption with C substance. The residual precipitate was washed with chilled water, centrifuged, and ground in a mortar with two other pooled low grade sera containing an additional 11.2 mg. of antibody N in 83 ml. After washing, followed by extraction with 15 per cent salt

TABLE II

Antibody Solutions from Dissociation of C-Anti-C Precipitates from Antipneumococcus Horse Sera

Serum and preparation number	Anti-C N taken	Anti-C N in recovered antibody solution	Recovery of anti-C N	Total N (per ml. of antibody solution)	Anti-C N (per ml. of antibody solution)	Anti-S III N (per ml. of antibody solution)	Total antibody N (per ml. of antibody solution)	Antibody N / Total N: 37°, 0.9 per cent NaCl extract	Antibody N / Total N: 15 per cent NaCl extract
	mg.	*mg.*	*per cent*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*
Type III									
792 CC_1	56	1.0	1.8	0.152	0.054	0.028*†	0.105	69	
CC_2		0.9	1.6	0.069	0.039	0	0.039(P)		57(P)
						Anti-S VIII N			
Type VIII									
644 C_1		2.3		0.248	0.041	0.191*‡	0.232(P)	94(P)	
C_2		1.1		0.153	0.054	0			35(P)
909 C_1	24	0.5	2.1	0.146	0.029	0.051*	0.080(P)	55(P)	
C_2		0.5	2.1	0.061	0.025	Trace*			41(P)

(P) after analyses indicates precipitin N only.

* After removal of anti-C.

† After removal of anti-C and anti-S III the supernatant gave 0.023 agglutinin N with Pn III.

‡ 0.071 mg. of this also reacts as anti-S III N.

solution (solution D), the residue was dissociated with barium hydroxide and barium chloride as in (1). For the next three solutions 36.5 ml. of serum 14.46, containing 2.12 mg. of anti-S I per ml., were absorbed with C substance, the residual anti-S I content being 1.78 mg. per ml. The serum was then diluted with 2 volumes of chilled saline and precipitated at 0° with eight successive 1 mg. portions of S I. The sixth washing at 0° and a seventh at 37° were combined and dialyzed against saline in the cold under negative pressure (solution F). An eighth washing, also at 37°, contained only traces of protein and was discarded. Both

the strong salt and barium hydroxide extracts of this specific precipitate yielded highly pure antibody (G and H), but repetition of the above procedure on a larger scale with a mixture of lower grade sera (K) gave a poor product. The remaining

TABLE III

Antibody Solutions from Dissociation of Specific Precipitates from Types I and III Antipneumococcus Rabbit Sera

Serum and preparation number	Antibody N taken	Precipitin + agglutinin N in recovered antibody solution	Recovery of antibody N	Total N	Precipitin N	Agglutinin N	Antibody N	Antibody N / Total N: 37°, 0.9 per cent NaCl extract	15 per cent NaCl extract	Ba(OH)$_2$ dissociated solution
				per ml. of antibody solution						
	mg.	*mg.*	*per cent*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*	*per cent*
Type I										
193 C	45.4	3.4	7.5	0.137	0.099	0.010	0.109		80*	
D	56.6	5.8	10.2	0.251	0.152	0.023	0.175		70*	
E		4.4	7.8	0.161	0.129	0.009	0.138			86
F	65.0	2.2	3.4	0.070	0.049	0.004	0.053	76†		
G		7.1	10.9	0.215	0.204	0.004	0.208		97	
H		9.8	15.1	0.269	0.251	0.000	0.251			93
K	177	12.8	7.2	0.976	0.495	0.121	0.616		63	
4.77 L	204	2.9	1.4	0.203	0.151	0.010	0.161	79		
M		20.0	9.8	1.224	0.833	0.159	0.992		81	
N		4.9	2.4	0.247	0.213	0.006	0.219			89
N′		17.0	8.3	1.054	0.897‡	0.048	0.945			90
4.75$_2$ P	85.2	0.4	0.5	0.039	0.010	0.004	0.014	36		
Q		1.1	1.3	0.056	0.041	0.004	0.045		80	
R		5.9	6.9	0.344	0.277	0.019	0.296			86
4.53 B	39.5	2.2	5.6	0.263	0.139	0.017	0.156		59*	
C		5.7	14.4	0.605	0.418	0.008	0.426			70
E§	34.3	0.6	1.8	0.088	0.041	0.012	0.053		63	
F		7.2	21.0	0.538	0.411	0.013	0.424			79
Type III										
199 A	102‖	14.0	13.7	0.551	0.461	0.016	0.477		87*	
B		2.6	2.5	0.133	0.105	0.000	0.105			79

* Precipitate not extracted first at 37° with saline.

† Sixth washing at 0° combined with a seventh at 37°C.

‡ One analysis discarded.

§ Precipitated at 37°.

‖ Approximate antibody N content.

solutions were prepared from high grade sera containing more than 2.0 mg. of antibody N per ml.[3] but the recovered antibody solutions were rarely more than 80 per cent pure. Possible reasons for this are given in the Discussion. Too little barium hydroxide was probably used for solution N, and the residue was accordingly dissociated once more with the same reagent (N′).

Table IV summarizes a rather comprehensive experiment with a bovine serum, of which nearly 1 liter was available.[4] The animal had been injected intravenously with a mixture of formalin-killed Types I, II, and III pneumococci, and the serum, sterile and without preservative, contained a total of 0.48 mg. per ml. of anti-S I, II, and III. Without previous absorption with C substance 860 ml. of the serum were precipitated at 0° with three successive 2.5 mg. portions of S I. The precipitate was centrifuged off (fraction B) and about 800 ml. of the supernatant were precipitated with three 2 mg. portions of S II (fraction C). The supernatant from this was precipitated similarly with 6 mg. of S III (fraction D). Since appreciable amounts of antibody, especially of anti-S III, remained in the supernatant, this was again precipitated with 2.1 mg. of S III (fraction D_4). The specific precipitates were washed thoroughly in the cold with saline and were then extracted successively with 0.9 per cent sodium chloride solution at 37°C. (B_1, C_1, D_1), 15 per cent sodium chloride solution at 37° (B_2, C_2, D_2), and barium hydroxide followed by barium chloride at 0° (B_3, C_3, D_3). Fractions C_3 and D_3 required larger amounts of barium hydroxide than usual, and much of the precipitate failed to dissolve, so that the greater alkalinity, coupled with longer exposure to alkali, may have been the cause of the unsatisfactory quality of the alkali-treated fractions. Slightly more than one-fifth of the precipitin in the serum was recovered in the various fractions. Fraction D_2 represents the only analytically pure antibody solution recovered thus far in the work, although it will be seen from the tables that others were obtained assaying 95 per cent and higher. Analyses in Table IV for heterologous type precipitins were made on the supernatants from the homologous precipitation; experiments in the reversed order are given in the footnotes to the table.

In Table V are given data on the purification of Type I pneumococcus anticarbohydrate formed in the pig and the sheep. Both sera were very weak, con-

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[3] Several rabbit antisera containing more than 4 mg. of anti-S I N per ml. were encountered in the course of the work. One of these contained 5.0 mg. of anti-S I N per ml. and 0.8 mg. of agglutinin N in the supernatant, or over 36 mg. of antibody protein per ml., an amount far higher than any previously reported (*cf.*, for example, 11, 17). This serum contained 14.0 mg. of N and 8.7 mg. of globulin N per ml. (Howe micro method), so that two-thirds of the globulin present, or over 40 per cent of the total protein, actually consisted of antibody.

[4] The material was supplied through the courtesy of Dr. John Reichel, Director of the Mulford Biological Laboratories of Sharp and Dohme, Glenolden, Pennsylvania.

TABLE IV

Antibody Solutions from the Dissociation of Specific Precipitates from a Bovine Type I, II, and III Serum

Serum 1224 D: Preparation	Precipitant	Antibody N taken (3 types)	Precipitin + agglutinin N in recovered antibody solution	Recovery of antibody N	Total N (per ml. of recovered antibody solution)	Anti-S I precipitin N (per ml. of recovered antibody solution)	Anti-S II precipitin N (per ml. of recovered antibody solution)	Anti-S III precipitin N (per ml. of recovered antibody solution)	Agglutinin N (per ml. of recovered antibody solution)	Antibody N (per ml. of recovered antibody solution)	Antibody N / Total N: 37°, 0.9 per cent NaCl extract	Antibody N / Total N: 15 per cent NaCl extract	Antibody N / Total N: Ba(OH)₂ dissociated solution
		mg.	mg.	per cent	mg.	mg.	mg.	mg.	mg.	mg.	per cent	per cent	per cent
B_1			4.7	1.1	0.299	0.223*	0.009	0.001		0.233(P)	78(P)		
B_2	S I	413	10.5	2.5	0.540	0.610	0.005†		0.007	0.522		97	
B_3			9.4	2.3	0.598	0.675			0.005	0.580			97
C_1			7.0	1.8	0.402	0.000	0.276			0.276(P)	69(P)		
C_2	S II	389	3.8	1.0	0.187	0.003‡	0.167	0.000		0.170(P)		91	
C_3			1.3	0.3	0.149	0.000	0.057	0.000	0.000	0.057			38
D_1			8.4	2.2	0.449		0.013§	0.402		0.415(P)	92(P)		
D_2	S III		23.2	6.0	1.007‖	0.000		1.007	0.003	1.010		100	
D_3			1.9	0.5	0.291**		0.002††	0.102		0.104(P)			36(P)
D_4‡‡			14.5	3.7	0.913			0.796§§		0.796(P)		87(P)	

(P) after analyses indicates precipitin N only.

* S I + S II together gave 0.225, S II first gave 0.036 mg. N per ml.

† S II first, followed by S I, gave resp., 0.013, 0.515 mg. N per ml.

‡ S I before S II gave 0.010 mg. N per ml.

§ S II, then S III gave 0.042, 0.369 mg. N per ml., resp.

‖ S II before S III gave 0.052 mg. N per ml; followed by S I, no N was precipitated.

** On determination only.

†† Anti-S I plus anti-S II.

‡‡ From precipitation of residual serum with additional S III.

§§ S II before S III gave 0.016 mg. N per ml; followed by S I, no N was precipitated.

taining only 0.31 and 0.12 mg. of anti-S I N per ml., respectively, so that a high degree of purity could scarcely be expected for the dissociated antibody. A purer end-product was obtained, using as starting material a Felton solution (14) prepared by pouring the pig serum into 30 volumes of 0.001 M phosphate buffer at pH 5, but the yield by this dual process was very poor.

3. Dissociation of Antibody from Specifically Agglutinated Pneumococci.—In this study Type I pneumococci and Type I antipneumococcus sera were used,

TABLE V

Antibody Solutions from the Dissociation of Specific Precipitates from Type I Antipneumococcus Pig and Sheep Sera

Serum and preparation number	Antibody N taken	Precipitin + agglutinin N in recovered antibody solution	Recovery of antibody N	Total N	Precipitin N	Agglutinin N	Antibody N	Antibody N / Total N: 37°, 0.9 per cent NaCl extract	Antibody N / Total N: 15 per cent NaCl extract	Antibody N / Total N: $Ba(OH)_2$ dissociated solution
				per ml. of antibody solution						
	mg.	*mg.*	*per cent*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*	*per cent*
Pig serum										
198 A	174	1.4	0.8	0.519	0.033	0.046	0.079	15		
B		12.5	7.2	1.920	0.317*	0.514	0.831		43	
C		3.6	2.1	0.353	0.186	0.049	0.235			67
AA	124	2.8	2.3	0.365		0.089	0.089	24		
BB		9.6	7.7	0.723		0.383	0.383		53	
CC		5.9	4.8	0.453	0.323	0.015	0.338			75
D†	124	24.4(P)	20	1.068	0.205					
E‡	23.8	4.4	18.5	0.307	0.187*	0.074	0.261		85	
Sheep serum										
202 A	96	2.4	2.5	0.945	0.054	0.114	0.168	18		
B		2.1	2.2	0.500	0.088	0.148	0.236		47	
C		0.6	0.6	0.078	0.040	0.005	0.045			58

* Insufficient S I used.

† Felton solution. 19 per cent of total N precipitable by S I.

‡ D used as starting material for dissociation.

since sera were available with which comparable experiments had been made on the dissociation of specific precipitates. Extension of the method to the purification of antibody of the other types is being undertaken.

Amounts of antiserum ranging from 10 to 680 ml. were diluted with an equal volume of saline, except in the case of the weak pig and sheep sera, of which the largest volumes were used. Freshly washed, heat-killed or formalin- and heat-treated Pn I suspension was added at 0°C. or at 37°C. in 2 to 25 ml. portions,

TABLE VI

Antibody Solutions Prepared by Dissociation from Pneumococci Agglutinated in Type I Antipneumococcus Sera

Serum and preparation number	Antibody N taken	Precipitin + agglutinin N in recovered antibody solution	Recovery of antibody N	Total N (per ml. of antibody solution)	Precipitin N (per ml. of antibody solution)	Agglutinin N (per ml. of antibody solution)	Antibody N (per ml. of antibody solution)	Antibody N / Total N: 37°, 0.9 per cent NaCl extract	Antibody N / Total N: 15 per cent NaCl extract	Antibody N / Total N: Ba(OH)$_2$ dissociated solution
	mg.	*mg.*	*per cent*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*	*per cent*
Horse										
701,2 A*	42.7	13.2	31	0.430	0.396†		>0.396(P)		>92(P)	
B		7.3	17	0.314	0.305		0.305(P)			97(P)
7032 A*		10.1		0.575	0.516	0.000	0.516		90	
B		1.8		0.173	0.151		0.151(P)			87(P)
Rabbit										
4.53 A*	56.8	0.8	1.4	0.084		0.039	0.039	46		
B		3.9	6.9	0.277	0.185	0.021	0.206		74	
C		22.1	39	1.318	1.125	0.040	1.165			88
4.56$_3$ A‡	70.4	6.5	9.2	0.305	0.242	0.014	0.256		84	
B		29.7	42	1.706	1.206†	0.400	1.606			94
C§	70.4	4.0	5.7	0.286	0.125	0.053	0.178		62	
D		26.7	38	1.374	1.154†	0.118	1.272			93
4.75$_2$ A*	54.0	0	0	0.026	0.000		0(P)	0(P)		
B		2.3	4.3	0.112	0.093		0.093(P)		83(P)	
C		24.2	45	1.466	1.422		1.422(P)			97(P)
D*	59.6	0.4	0.7	0.067		0.023	0.023	34		
E‖		2.2	3.7	0.222		0.150	0.150		68	
F		24.5	41	1.296	1.130	0.035	1.165			90
Pig										
198 F‡	62	15.3	25	0.802	0.499	0.078	0.577		72	
G		2.1	3.4	0.102	0.063	0.013	0.076			75
Sheep										
202 D§	82	5.1	6.2	1.177	0.128	0.105	0.233		20	
E		6.1	7.4	0.407	0.237	0.052	0.289			71

(P) after analyses indicates precipitin N only.

* Agglutination carried out at 37°, washed at 0°C.

† Insufficient S I used.

‡ Agglutination and washing carried out at 37°C.

§ Agglutination and washing carried out at 0°C.

‖ Differed from 4.75$_2$ B only in use of mechanical stirrer during 15 per cent salt extraction at 37° for 3 hours.

depending on the bacterial N content, which varied from 2.8 to 0.4 mg. per ml. Addition of the Pn I was continued, with frequent centrifugation, until agglutination of the added cells occurred only slowly, or until the supernatant gave only a faint precipitin test with S I. The agglutination complex was thoroughly washed with saline at the appropriate temperature. After three to seven washings, the supernatant usually contained only traces of heat-coagulable protein. In some of the 0° experiments the material, after washing at 0°, was given one or two additional washings at 37°C. and analyses were made of the resulting solution after concentration by dialysis against saline under negative pressure.

For the extraction of the washed residue it seemed most advantageous to smooth out all lumps during the addition of 10 to 30 ml. of 15 per cent sodium chloride solution, allow the mixture to stand overnight at 37° in the presence of a little toluene, centrifuge, and then wash once at 37° with one-third to one-half of the initial amount of 15 per cent salt solution. An inferior product resulted in the one instance in which mechanical stirring was used. The antibody solutions were dialyzed and concentrated as in section 2. The extracted residues were submitted to dissociation by barium hydroxide and barium chloride (using 4 ml. of 10 per cent solution of the latter) in the cold as in the case of the specific precipitates. The properties of the resulting solutions are summarized in Table VI.

Preparation 701,2 was a mixture of 47 ml. of absorbed serum 701, 12 ml. of serum 702, and 11 ml. of a Felton solution B 78, the properties of which had been described in (3). Preparation 7032, of unknown antibody content, was absorbed with C substance before dilution and use. Although the rabbit sera were not absorbed with C substance before addition of the Pn I the only dissociated antibody tested, 4.75_2C, contained no anti-C. The data obtained with serum 4.56_2 indicate that purer antibody may be dissociated from Pn I agglutinated in rabbit serum at 37° than from cells agglutinated at 0°, although it is probable that in this case also, a preliminary washing with saline at 37°, as in 4.75_2 A and B, would have improved the antibody subsequently dissociated by means of strong salt. In all of the rabbit sera the yields from the salt dissociation of the agglutination complex were small, while the recovery by the barium hydroxide method was extraordinarily high. Antibody recovery at 0° from the very weak sheep antiserum was disappointing.

DISCUSSION

In the first report (1) on a theoretically indicated procedure (2) for the dissociation of precipitates formed by pneumococcus specific polysaccharides and homologous antisera it was shown that antibody assaying over 85 per cent of immunologically reactive material was readily obtainable in a single step from Types I and II antipneumococcus horse sera and Type III antipneumococcus rabbit sera. It is now found that when the initial specific precipitates are adequately

washed Type III antipneumococcus horse sera also yield antibody (anticarbohydrate) solutions in which 92 to 98 per cent of the nitrogen present is immune nitrogen. In two of these solutions (792 N and P, Table I) the anticarbohydrate appeared to be entirely in the native state, since it was quantitatively removable as precipitin, as in untreated sera (18, 1), leaving no residual agglutinin. The purest solution, 792 T, was obtained by dissociation with strong salt after precipitation and wasbing of the serum at 0°C. and a preliminary extraction with 0.9 per cent salt solution at 37°C. It is probable, from the analysis of sample 792 P, that solutions of equal purity could be obtained by carrying out both the precipitation and washing at 37°, although in this case a portion of the total antibody would remain unprecipitated (*cf.* 12).

It was also found possible to attain the same degree of purity with the cross reacting anticarbohydrate precipitated by S III from two different Type VIII antipneumococcus horse sera (Table I). In both of the solutions obtained by dissociation with strong salt 97 per cent of the precipitin present (anti-S VIII) was specifically precipitable by S III, which had been used for the initial precipitation. It is thus sbown that the Type VIII antisera contain a fraction of antibody which reacts practically as completely with S III, the heterologous cross reacting polysaccbaride, as with S VIII, the bomologous specific carbohydrate. An attempt will be made to obtain enough of this antibody for study, as it should afford a closer insight into the factors involved in this instance of cross reactivity (19).

While the dissociation of the C-anti-C precipitates from Types III and VIII antipneumococcus borse sera yielded little antibody and that of low purity from the standpoint of anti-C content, the solutions showed several features of interest (Table II). Two of the three 0.9 per cent saline extracts made at 37° after precipitation and washing at 0° contained more type specific antibody than anti-C. Tbus the precipitin nitrogen, anti-C N plus type specific N, of one of the solutions, 644 C_1, was 94 per cent of the total nitrogen although the anti-C content was only 17 per cent of the total. Since the saline extract therefore contained almost pure precipitin, the anti-S VIII could scarcely have been adsorbed non-specifically on the C precipitate, but was presumably attached owing to a low degree of immunological

and therefore chemical cross reactivity, possibly in much the same way as the fraction of low grade antibody precipitated from many sera when an excess of antigen is added in one portion, but left behind in the serial precipitation of antibody (*cf.*, for example, 17*a*). In favor of this view is the somewhat higher proportion of precipitin reactive with S III in this extract than in the original serum, and also the failure of similar extracts of C-anti-C precipitates derived from Type I antipneumococcus rabbit sera to show more than traces of anti-S I. The three 15 per cent salt extracts contained only anti-C and no appreciable amounts of anti-S III or anti-S VIII, so that the type specific antibody appears to be bound by the precipitate only at 0° and to be given off entirely on washing with 0.9 per cent saline at 37°. This is in accord with the finding of Goodner and Horsfall (20 *c*) that cross reactive antibody precipitable at 0°, as we had found (1), does not come down with the homologous specific precipitate at 37°. However, the assumption made by these workers that the precipitated cross reactive antibody is held by adsorption is not sustained by the evidence just presented.

With Type I antipneumococcus rabbit sera it was found (Table III) that occasional sera yielded highly pure antibody by the salt dissociation method as readily as did the Type III rabbit sera previously studied (1). Other sera, whether precipitated at 0° or at 37° and regardless of their antibody content, furnished in poor yield antibody solutions in which not more than one-half to three-quarters of the nitrogen was immune nitrogen. Characteristic, also, of some of these solutions was the relatively high proportion of antibody which could not be precipitated by S I but was recoverable as agglutinin, even in the case of sera which had previously been absorbed with C substance.

A possible reason for the relatively unsatisfactory antibody recovery on dissociation of the Type I rabbit precipitates may be the low antibody:S I ratios in these precipitates (21). On account of their high content of S I the shift in equilibrium between antibody and S I on the addition of strong salt (1, 2) might not be as extensive as in the case of other specific precipitates containing less polysaccharide, resulting in poor yields and a relatively higher proportion of impurities. Application of the barium hydroxide modification (1) of Felton's dissociation method (7) to the salt-extracted agglutination

residues gives particularly good results both as to yield and analytical purity (see Table VI).

Appended to Table III are data on antibody solutions obtained directly from low grade, pooled Type III anti-Pn rabbit sera, showing that both dissociation methods may be applied even to sera of low antibody content.

The data in Table IV not only indicate the utility of the salt dissociation method for the purification of antibodies in a low grade bovine anti-Pn serum, but show that, in spite of the injection of the animal with Types I, II, and III pneumococci, antibody to each type was separately recovered in an exceedingly high state of purity. Since the type specific antibodies were separately precipitable from the serum by the appropriate polysaccharide, and the dissociated antibody of each type showed little cross reactivity with the other types, it is again evident (*cf.* 1) that in a polyvalent serum most, if not all, of the antibodies to each pneumococcus type occur as molecules distinct from those of the other types. The data also provide the first instance of the preparation, from raw serum without previous concentration, of analytically pure antibody (solution D_2).

Even though analytically pure antibody has been prepared, it cannot be considered as a single chemical entity, for earlier work has shown that pneumococcus anticarbohydrate (15, 1) and even antibody to a single crystalline antigen such as egg albumin (17*a*, 23) consist of a separable mixture of antibodies of differing reactivity.

Initial experiments on low grade pig and sheep sera (Table V) were less encouraging. It was found, however, that preparation of a Felton solution from the pig serum as a preliminary step (198 D) permitted the preparation of antibody of a high degree of purity (198 E).

The salt dissociation of agglutinated Pn I proved so satisfactory and simple of execution that it would seem to be the method of choice for the rapid preparation from antipneumococcus sera of highly pure antibody solutions, especially if the agglutination be carried out at 37°C. (Table VI). Horse antisera (without preliminary concentration) readily yielded solutions in which 90 per cent of the nitrogen was recoverable by precipitation with S I. With the rabbit antisera the salt-dissociated antibody was at least as pure as that dissociated

from specific precipitates, and the yield appeared somewhat better. A direct comparison is afforded by the data on serum 4.75_2 in Tables III and VI. In all of the rabbit antisera between one-third and one-half of the total serum antibody could be recovered from the agglutination complex remaining after salt dissociation by use of the barium hydroxide-barium chloride method. Not only were the yields extremely high, but the analytical purity of the resulting antibody ranged from 88 to 97 per cent. Applied to the pig serum the agglutination procedure gave a 25 per cent yield of salt-dissociated antibody of a higher degree of purity than given by the precipitin method (Tables V and VI). The result with sheep serum at 0°, the only temperature used, was not as good.

Although the dissociation of agglutinated Type I pneumococci gave solutions equalling or exceeding in yield and analytical purity those from the corresponding treatment of specific precipitates, and the procedure offers the additional advantage that isolation of specific polysaccharide is unnecessary, it must be borne in mind that antibody solutions prepared from agglutinated pneumococci, especially those dissociated by the alkaline barium hydroxide method, may contain pneumococcus protein. While in many of the solutions this does not seem to be present in sufficient amounts to affect the analytical results, its occurrence might be of biological importance. It is planned to test these solutions for bacterial protein.

Confirmation of the identity of pneumococcus anticarbohydrate agglutinin and precipitin (*cf.* 18) by an independent method is also afforded by several of the experiments summarized in Table VI. It will be noted that in solutions 701,2 B, 7032 A, 4.75_2 C, and 4.75_2 F, 97 to 100 per cent of the antibody nitrogen present was precipitable by S I. Since this antibody had originally been removed from horse and rabbit sera as agglutinin its quantitative recovery as precipitin again shows that the only difference between anticarbohydrate agglutinin and precipitin is a difference in the distribution and state of the pneumococcus polysaccharide serving as a reagent for both: in the case of agglutinin, the polysaccharide reagent is bound to the pneumococcus cell; in the case of precipitin the polysaccharide exists free in solution. The correlation between precipitin and protective antibody (10, 24) is not as simple, especially with antibody produced

in the horse, as Goodner and Horsfall (20) have recently shown. In this added respect they have confirmed our laboratory's finding that pneumococcus anticarbohydrate, especially that produced in the horse (22, 15, 19), is not a single substance, but a series of substances of differing degrees of reactivity.

The generally high proportion of antibody in the 0.9 per cent saline, 37°C. extracts of specific precipitates or agglutinated pneumococci prepared at 0° is in accord with observations that more antibody is analytically demonstrable in pneumococcus antisera at 0° than at 37° (12, 15, 21, 3). Moreover, the dissociation of this fraction of the antibody in physiological saline at 37° is predictable if specific precipitation and agglutination are regarded as reversible chemical reactions (15, 3).

It has frequently been assumed that in specific precipitation and agglutination the antibody taking part is denatured. It has been shown, however, that these immune reactions may be qualitatively (25) and quantitatively (15, 17*a*, 3, 26) accounted for without making this assumption, and, indeed, there would seem no experimental foundation for its use. Nevertheless, Chow and Wu (6) have used the reversal of an assumed denaturation as the explanation of the recovery of antibody from S-anti-S precipitates and agglutinated pneumococci by their modification of Felton's alkali dissociation procedure (7). While experimental verification of this may eventually be forthcoming, Chow and Wu have claimed "immunological purity" for their recovered antibody on the basis of a correction for the solubility of specific precipitates formed from the antibody. That such a correction is inadmissible is shown by the virtual insolubility at the temperature of precipitation of S-anti-S precipitates from horse sera (15), the lower solubility of corresponding precipitates from rabbit sera (21) than that used for the correction by Chow and Wu, and the frequent preparation in this laboratory (1, and the present work) and by Goodner and Horsfall (20) of antibody solutions showing an analytical purity of 97 to 100 per cent without any deduction for solubility. One such solution, prepared by alkali dissociation, was examined in the ultracentrifuge (27) and showed evidence of molecular degradation in spite of the high degree of analytical purity. If degradation too slight to affect the precipitating value could be caused

by the brief exposure of the specific precipitate to alkali used in the present studies, the longer alkali treatment employed by Chow and Wu might possibly necessitate a correction for impurities due to greater degradation, but such material could not be called pure antibody.

SUMMARY

1. The salt dissociation and barium hydroxide-barium chloride methods are extended to the preparation of highly purified antibody solutions from specific precipitates derived from Type III and Type VIII antipneumococcus horse sera and a low grade polyvalent bovine serum. Analytically pure precipitin (agglutinin) was obtained from the last, and Types I, II, and III antibodies were separated.

2. Difficulties connected with the application of the methods to Type I antipneumococcus rabbit sera are described, as is also the purification of antibody from low grade pig and sheep sera.

3. The dissociation of antibody by both methods from Type I pneumococci agglutinated in antisera produced in the horse, rabbit, pig, and sheep, is described and its advantages discussed.

4. Certain theoretical aspects of the work are also discussed.

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INFLUENCE OF HOST FACTORS ON NEUROINVASIVENESS OF VESICULAR STOMATITIS VIRUS

III. Effect of Age and Pathway of Infection on the Character and Localization of Lesions in the Central Nervous System

By ALBERT B. SABIN, M.D., and PETER K. OLITSKY, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

Plates 6 to 9

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The experimental data of the preceding investigations (1, 2) suggested that the failure of older animals, which readily succumb after intracerebral injection of the virus, to exhibit any clinical manifestations of central nervous system (CNS) involvement after peripheral inoculations was possibly due to the presence of certain barriers to the invasiveness of the virus, encountered at the site of inoculation when the intramuscular or intraocular routes were employed and within the CNS when the virus was given by way of the nose. The hypothesis of localized barriers developing with increasing age of the host depends to a great extent on evidence that the distribution or movement of this virus in the nervous system of mice occurs by different mechanisms after peripheral and intracerebral inoculations. The purpose of the present investigation was to determine whether a detailed study of the localization of lesions in the nervous systems of susceptible and resistant mice after different modes of inoculation could throw additional light on the movement of the virus and thus on the nature of the resistance of older animals.

If the movement of vesicular stomatitis virus in the nervous system of mice occurs along the pathways suggested by the preceding experimental studies, one would expect that the distribution of the lesions would vary with intracerebral and peripheral inoculation, and that after peripheral injection they would also vary with the central connections of the nerves along which the virus gained

entrance. The successful demonstration of such specific variations in the localization of CNS lesions could add not only to the evidence for the axonal and trans-synaptic movement of this virus but also to the hypothesis of localized barriers along such a path. If, on the other hand, the distribution of the lesions should prove to bear no relationship to the route of inoculation or not to depend upon the central connections of the neurons supplying the sites of peripheral injection, then the above concepts would become untenable.

Histopathological studies have been successfully employed by a number of investigators (Goodpasture and Teague (3); Marinesco and Draganesco (4); Pette (5); Hurst (6); Sabin and Hurst (7)) to show that with certain viruses the primary lesions in the CNS are determined by the peripheral nerve supply of the inoculated area. The relation of secondary and subsequent localizations of lesions in the CNS to the known central connections of the site of primary attack has been studied but little. Seifried and Spatz (8) recently stated that the spread of the viruses of poliomyelitis, "epidemic encephalitis," rabies, and Borna's disease within the CNS was by way of the spinal fluid. Fairbrother and Hurst (9) and Hurst (10) believed from their studies with the virus of poliomyelitis that both after intracerebral and intrasciatic inoculation the localization of secondary lesions within the CNS was determined by the tract connections of the site of primary attack. With equine encephalomyelitis, however, Hurst (11) stated that in the guinea pig "lesions were substantially the same whether the virus was introduced intracerebrally, intramuscularly, subcutaneously or intradermally," and in a subsequent communication (12), that the localization within the CNS was frequently essentially the same after intranasal as after intramuscular inoculation.

Methods

The mouse is particularly suited for this type of study because (*a*) its size readily allows sectioning of almost the entire CNS, and (*b*) in an investigation such as this it is important to determine not only that lesions are present in certain definite regions but also that they are absent in others.

In the beginning most of the work was done with brains taken out of the cranial cavity and fixed in Zenker's fluid containing 5 per cent glacial acetic acid. Later on, it was found that satisfactory fixation occurred through the bone, so that the entire head minus the skin and lower jaw was fixed in the Zenker-acetic mixture for 24 hours. The acid caused sufficient decalcification to permit the sectioning of all the structures, including the nasal mucosa, eyes, and cranial ganglia, *in situ*. In a few instances sections were cut parasagittally, extending from the nose to the medulla. As a rule, however, the entire skull was embedded (paraffin) with the anterior end down, the sections being cut serially in the frontal transverse plane, at a thickness of 5 to 6μ. Ribbons of four to six sections out of each twenty to thirty were taken for staining. The entire spinal column was fixed in the same manner so that sections at various levels showed not only the cord, roots, and

spinal ganglia but frequently also the sympathetic ganglia. The sections were stained with phloxine and methylene blue, eosin and methylene blue, or hematoxylin and eosin.

Remarks on Spontaneous Encephalitic Lesions in Mice

Pathological investigations on the CNS of rabbits were often confusing until the occurrence of spontaneous, asymptomatic, encephalitic lesions generally referred to as being caused by *Encephalitozoon cuniculi* was recognized. That similar difficulties complicate studies on the CNS of mice has not as yet been sufficiently appreciated. Cowdry and Nicholson (13) found meningoencephalitic lesions in the brains of twenty-five of 132 mice studied, and in five of the twenty-five positive cases they noted the coexistence of protozoan-like parasites. Smadel and Moore (14), in their report on the pathology produced by the virus of St. Louis encephalitis in mice, also noted the presence in some of their animals of these spontaneous encephalitic lesions. In the present investigation it was observed that the occurrence of spontaneous meningoencephalitic changes in the Rockefeller Institute mice was distinctly related to the age of the animal; while none was detected in about fifty young mice (less than 1 month old), practically all the old animals (about 9 to 12 months of age) showed these lesions when many sections of the same brain were examined. The pathology consisted of dense perivascular cuffs of small, round cells, meningeal infiltration with similar cells, and small glial and occasionally granulomatous foci in the brain substance. These lesions could be found scattered irregularly throughout the CNS from the tips of the olfactory bulbs all the way down to the sacral cord. Sometimes, however, many sections of the same brain had to be examined before one or the other of these changes was found. Although no protozoan-like parasites were observed in any of the present sections, one cannot exclude the possibility of their having been in the brains at some previous time. In view of the fact, however, that the spontaneous virus encephalomyelitis described by Theiler (15) is endemic in the Rockefeller Institute stock (1),[1] one must consider the possibility that some of these chronic meningoencephalitic reactions (particularly those which do not show granulomatous foci) may, perhaps, represent the residual lesions of a subclinical attack with this virus.

General Aspects of Vesicular Stomatitis Virus Lesions

In the CNS, primary necrosis of nerve cells is the outstanding lesion. The process is acute and the reaction to it consists chiefly of an invasion of leucocytes into the necrotic zones. Perivascular cuffing is either absent or extremely rare and inconspicuous; only occasionally does one find a vessel surrounded by a single layer of polymorphonuclear and mononuclear cells. There is no definite evidence

[1] In the past 2 years the incidence of the apparent form of this type of encephalomyelitis was found to be 1 or 2 per 1000.

of a direct attack of the virus on the meninges after intracerebral or peripheral inoculation. There is, however, some infiltration of the meninges with mononuclear and polymorphonuclear cells, representing a response to injury of the nervous tissue, although the distribution of the exudate bears no particular relationship to the presence or absence of cerebral changes in a particular region. There is no difficulty in distinguishing the rather mild, acute, inflammatory reaction in the virus lesions from the diffusely scattered, chronic, and intense perivascular and meningeal infiltration encountered in the spontaneous cases. Small, round, acidophilic, intranuclear "inclusions" have been described as occurring in nerve cells affected by vesicular stomatitis virus (16). In the present study these were found so rarely as to be of no practical aid in mapping out sites affected by the virus. A more frequent change preceding the complete disintegration of the cell consists of a marked increase in the nuclear acidophilic material which shrinks from the membrane, giving rise to a clear halo. The nucleolus, however, remains *in situ*, and there is no margination of the basophilic material. This picture resembles nerve cell changes in mice injected with yellow fever virus (17), and with other viruses in nerve cells of other hosts, but does not appear to us to have the characteristics of a specific inclusion body.

Pathology after Intracerebral Injection

Young Mice.—The CNS of three mice, 15, 21, and 30 days of age, were examined. Mice of this age die within less than 48 hours after intracerebral injection of 100 or more minimal infective doses. When many sections are made the site of inoculation can be recognized as a tract of necrotic cells extending through the neopallial cortex and into the underlying diencephalon or mesencephalon. The lesion is sharply demarcated and appears to correspond roughly to the size of the injecting needle. From the site of inoculation, lesions are found to extend anteriorly and posteriorly in close relation to the ventricular system and its extension anteriorly, the rhinocele, and posteriorly along the ependyma of the central canal.

The brain of the 15 day old mouse showed no changes anterior to the lateral ventricles. In the other brains, the olfactory bulbs exhibited extensive necrosis, most marked in and about the rhinoceles and involving the cells of the internal granular and mitral layers. The outer layers of the bulbs (external granular, glomerular, and layer of nerve fibers) appeared generally well preserved, suggesting that the spread of the lesions was from the rhinocele outwards. Longitudinal and partial transverse serial sections indicated that the lesion was a continuous one, extending from the lateral ventricles to the tips of the olfactory bulbs. At the level of the lateral ventricles there was evidence of involvement of the ependymal cells as well as necrosis of a few layers of periventricular nerve cells, more marked ventrally but occurring also at the sides and dorsally in the corpus callosum. Anterior to the optic chiasm there were no other discernible lesions and the meninges showed no evidence of being attacked by the virus or of having permitted it to pass through it to the underlying brain tissue. Beyond the level

of the optic chiasm the periventricular distribution of the lesions was again apparent. There was necrosis of the cornu Ammonis where it forms the floor and inner sides of the lateral ventricles, as well as of the parts of the neopallial cortex which form the roof and outer sides. These changes did not extend for any appreciable distance beyond the ventricular walls. With the exception of a few necrotic cells and some polymorphonuclear leucocytes in the habenular nuclei, there was little involvement of the tissues surrounding the third ventricle. In one case (15 day old mouse) there was a considerable invasion of tissues around the third ventricle with polymorphonuclear leucocytes but without any evidence of cellular necrosis. Again with the exception of a small area of necrosis in the tectum of one brain, apparently forming a part of the needle tract, there were no significant lesions in the midbrain.

The meningeal reaction was slight in two instances, consisting chiefly of the infiltration of polymorphonuclear and a few mononuclear leucocytes, and rather marked in the third which exhibited an exudate containing a great deal of fibrin and polymorphonuclear leucocytes, particularly around the midbrain and in the region of the dorsal portion of the third ventricle. After intensive study this severe reaction appeared to be due neither to an attack of the virus on the meninges themselves nor on the underlying brain tissue, but rather to the trauma of inoculation.

Continuing posteriorly, one found an occasional necrotic focus in relation to the fourth ventricle, and definite involvement of the ependyma of the central canal and the contiguous nerve cells. Strangely enough the lesion was very slight in the cervical and thoracic spinal cord, but quite extensive in its lumbar portion.

No lesions were found in any of the following structures connected with the CNS: the sensory ganglia of the cranial nerves, the spinal ganglia, the submaxillary, ciliary, otic, and superior cervical sympathetic ganglia, the hypophysis, pineal body, retina, and nasal mucosa.

In summary it can be stated that after intracerebral injection of vesicular stomatitis virus in young mice the recognizable CNS lesions present shortly before death of the animal are situated along the ventricular system and its extensions in the brain, and along the central canal of the spinal cord. The indications are that the primary spread of the virus occurred along this open pathway. There is no evidence that the meninges are attacked by the virus (not even at the site of inoculation), nor that virus spreads along the subarachnoid space.

Old Mice.—It has already been indicated that old mice (about 8 months to 1 year of age) and young mice are equally susceptible to intracerebral inoculation of vesicular stomatitis virus in the sense that the minimal infective dose is the same

for both, but that the old mice develop signs and succumb a day or two later than the young ones. The brains of two intracerebrally injected, 1 year old mice, sacrificed when prostrate and near death, were studied. The CNS of the mouse sacrificed 4 days after inoculation showed no lesions which could be attributed to the effect of the virus. The changes present corresponded entirely to those of "spontaneous encephalitis" encountered in uninoculated old mice. The CNS of the mouse sacrificed 3 days after inoculation also showed the lesions of spontaneous encephalitis with several granulomatous nodules in one olfactory bulb and widespread lymphocytic perivascular and meningeal infiltration, but there were, in addition, two slight and very sharply limited lesions of the type encountered in the young mice. One of these was in one of the olfactory bulbs and consisted of an area of acutely necrotic nerve cells infiltrated with polymorphonuclear cells, extending from the rhinocele up to and including a few cells of the mitral layer. Sections of the same olfactory bulb just anterior or posterior to this area appeared entirely normal. The other zone was ventral and lateral to the fourth ventricle and consisted of a few necrotic nerve cells and a few polymorphonuclear cells.

It appears from these data that the nerve cells of the old mice are generally more resistant to necrosis than are those of the young animals, in spite of the fact that sufficient multiplication of virus and change occur in these cells to give rise to signs of encephalitis and death. This relative absence of significant lesions is even more remarkable since the infective process lasts twice as long in the old as in the young mice.

Pathology after Nasal Instillation of Virus

Young Mice.—Nine mice (seven 15 days and two 21 days old) were studied. Mice of this age succumb 4 or 5 days after nasal instillation of virus. Two 15 day old animals were sacrificed 2 days after instillation when they still appeared entirely well. The sections through the nasal mucosa did not permit any definite conclusion about the state of the cells in the olfactory or respiratory mucosa. There was, however, neither necrosis nor inflammation. The olfactory nerve roots in the mucosa and at their junction with the olfactory bulbs showed no inflammatory or other visible change. The meninges over the olfactory bulbs and the rest of the brain appeared entirely normal, as did the nerve tissue itself, with the possible exception of some of the mitral cells in the olfactory bulbs. These cells showed what may perhaps represent the early changes of virus action.

The material from one of the remaining seven mice was obtained immediately after death, while the others were sacrificed either when they showed pronounced nervous signs or when prostrate and near death. While there was a certain amount of individual variation in the extent and location of lesions, their dis-

TABLE I

Distribution of Necrotic Lesions in Various Regions of the Central Nervous Systems of Mice Succumbing after Nasal Instillation of Vesicular Stomatitis Virus

Region	Age of mice and remarks					
	15 days old			21 days old		1 yr. old
	Dead 4½ days (no circling)	Sacrificed 4th day (circled left)	Sacrificed 4th day (no circling)	Sacrificed 5th day (circling)	Sacrificed 5th day (no circling)	Sacrificed 8th day (no circling)
Respiratory mucosa	–	–	–	n.s.	n.s.	n.s.
Olfactory mucosa	++	+++	+++	n.s.	n.s.	n.s.
Olfactory bulbs	+++	++++	++++	+++	+++	++
Lateral olfactory gyrus	–	+	++++	–	±	+ (unilateral)
Septum (ventromedial aspect)	–	–	++	–	–	+++
Tuberculum olfactorium	+++	+++	+++	+++	++++	+
Piriform lobe (including amygdaloid complex)	–	–	+++	–	–	++ (unilateral)
Cornu Ammonis	–	–	++	–	–	+
Hypothalamus (tuber cinereum)	+++	+	+++	++	+++	+
Thalamus	–	–	?	–	–	±
Mammillary body	+	+	+	+	+	±
Habenular nuclei	–	–	++	–	+	±
Interpeduncular nucleus	–	–	++	–	+	+
Lateral and medial geniculate bodies	–	–	–	–	–	–
Superior and inferior colliculi	–	–	–	–	–	–
Tegmental nuclei, anterior	± (red nucleus)	–	–	–	–	+++
Gudden's and other posterior tegmental nuclei	+++	+	+++	++	+++	++++
Nuclei of pons	–	–	–	–	–	–
Neopallial cortex	–	–	–	–	–	–
Corpus striatum	–	–	–	–	–	–
Sensory nucleus of fifth nerve	–	–	–	–	–	–
Motor nucleus of fifth nerve and reticular formation	+	+	++	+	–	+++
Vestibular nucleus of eighth nerve	–	++ (left side)	–	++	–	–
Deiters' and Bechterew's nuclei	–	++ (left side)	–	+	–	–
Cerebellum—deep nuclei	–	–	–	+	–	–
—rest of medula and cortex	–	–	–	–	–	–
Spinal cord	–	–	n.s.	n.s.	±	–
Gasserian ganglia	–	–	–	n.s.	n.s.	n.s.
Superior cervical sympathetic ganglia	–	–	–	n.s.	n.s.	n.s.
Submaxillary ganglia	–	–	–	n.s.	n.s.	n.s.

n.s. = no sections.

± = necrosis of occasional cell, found with difficulty.

\+ = small necrotic focus present in only a few of a series of sections from the same region.

++ = limited zone of necrosis present in most sections of series from same region.

+++ = extensive necrosis seen in all sections of region.

++++ = almost complete necrosis of region.

– = no evident neuronal lesion.

tribution corresponded to so definite a plan that they can all be described together. Some idea of the variations can be obtained from Table I, in which the presence or absence of lesions in certain regions of the CNS of five young mice is indicated.

The olfactory mucosa constitutes the greater portion of the nasal membrane of mice and it appeared to be primarily attacked by the virus. The lesion manifested itself as a necrosis of scattered patches of olfactory mucosa with little or no inflammatory response. At this stage many areas were denuded of cells, the necrotic debris lying free in the nasal cavities. No definite evidence could be found of involvement of the respiratory mucosa, although the occasional presence of suspicious intranuclear acidophilic dots was somewhat confusing. No lesion of any kind was found in the roots of the olfactory nerve in the mucosa, nor as a matter of fact in the outer layer of the olfactory bulbs consisting of the lamina fibrorum nervi olfactorii. The chief lesion in the olfactory bulbs was in the mitral cell layer, where a varying number or practically all the cells may be necrotic Frequently there were only empty spaces left where the mitral cells had been present originally. Depending upon the severity of the process, there was necrosis of the cells in the internal granular and gelatinous layers, and only rarely in the cells of the external granular layer surrounding the glomeruli which, as a rule, were entirely spared. The distribution of lesions in these olfactory bulb sections was quite different from that seen after intracerebral injection. After nasal instillation of virus the mitral cells appeared to be the center of the lesions and, even when practically the entire bulb was necrotic, the rhinocele remained undamaged, while after intracerebral injection it was clear that it was from this structure that the lesion extended.

Beyond the olfactory bulbs where the neopallial cortex joins the rhinencephalon, the former appeared entirely normal and in the latter there was usually a zone of varying extent which showed no lesions until the region of the tuberculum olfactorium and anterior perforated space was reached. Here there was a varying amount of necrosis in every case, while the lateral olfactory gyrus showed definite necrosis in only one and involvement of a very small patch of cells in two others; the septal region was involved in only one case. There were no lesions whatever around the rhinocele or the lateral ventricles, nor was there necrosis of tissue adjacent to the meninges. The lesions just described were practically always separated from either the ventricles or the meninges by normal appearing tissue.

Posterior to the optic chiasm the further distribution of necrotic foci appeared to depend upon the position of the lesions anterior to it. Thus, the cornu Ammonis was unaffected in all but one instance, and in that instance there was also necrosis in the ventromedial aspect of the "septum." Again, in only one case was there necrosis in the piriform lobes, and then it was unilateral and on the same side as extensive involvement of the lateral olfactory gyrus. The tuber cinereum of the hypothalamus was affected in all mice and some slight necrosis could always be found in the mammillary body. The habenular nuclei showed foci of necrosis in two mice. No lesions were found in the thalamus or other diencephalic structures. The tectum of the midbrain was negative in all cases, as was the anterior

portion of the tegmentum, with the possible exception of a few necrotic cells in one red nucleus of one brain. The interpeduncular nucleus showed necrosis in two cases and it is to be noted that it occurred only in the brains with involvement of the habenular nuclei. There were always, however, lesions in the posterior part of the tegmentum in the region of Gudden's nucleus and to a varying extent ventrally in the nuclei of the raphé and occasionally laterally, involving the mesencephalic nucleus of the fifth nerve. The nuclei of the pons exhibited no lesions. In the medulla there was necrosis in one or both motor nuclei of the fifth nerve and in varying regions of the formatio reticularis in all but one case.[2] There was never any evidence of involvement of the sensory nucleus of the fifth nerve. In two instances which corresponded with circling as the outstanding clinical sign, there was unilateral necrosis in the area of the vestibular nucleus of the eighth nerve, and the region of Deiters' and Bechterew's nuclei. In one of these brains, the nuclei of the cerebellum showed necrosis, while in all other instances the cerebellum appeared normal. The neopallial cortex was never involved. The spinal cord was examined in three cases and, with the exception of a few polymorphonuclear and necrotic nerve cells, there were no significant changes.

Special attention was paid to the nuclei of the other nerves supplying the nasal mucosa but no lesions were found in the Gasserian (sensory fifth), submaxillary ("parasympathetic"), or superior cervical (sympathetic) ganglia. It may also be stated that lesions were seen neither in the other sensory or autonomic cranial ganglia nor in the spinal ganglia. The lungs, liver, spleen, kidneys, and suprarenals of mice sacrificed on the 2nd and 4th days of the disease showed no changes attributable to the action of the virus.

Relation between Central Connections of Nerve Supply of Nasal Mucosa and Distribution of Lesions.—From the foregoing description, it is evident that of the various nerves connected with the nasal mucosa (sensory fifth, sympathetic and parasympathetic fibers, and olfactory nerves[3]) *only* the olfactory pathway showed signs of having been traversed by the virus. The choice of this special pathway by vesicular stomatitis virus cannot be regarded merely as a natural consequence of special anatomical relations (the large number of exposed olfactory neurons and possibly other direct connections with the CNS), since, as will be shown in another communication, not all viruses given intranasally to mice of the same breed and age invade the CNS along the olfactory nerves, but select instead the other nerves of the nasal mucosa. The use of the olfactory pathway should, perhaps, be regarded, therefore, as the result of some special affinity of vesicular stomatitis

[2] With regard to the involvement of the various regions in the midbrain, pons, and medulla reference may be made to Wallenberg's studies on the ramifications of the basal olfactory tract or bundle in the rabbit. (Wallenberg, A., *Anat. Anz.*, 1901–02, 20, 175.)

[3] This does not include the small nervus terminalis about which relatively little is known and which was not readily distinguished in our sections.

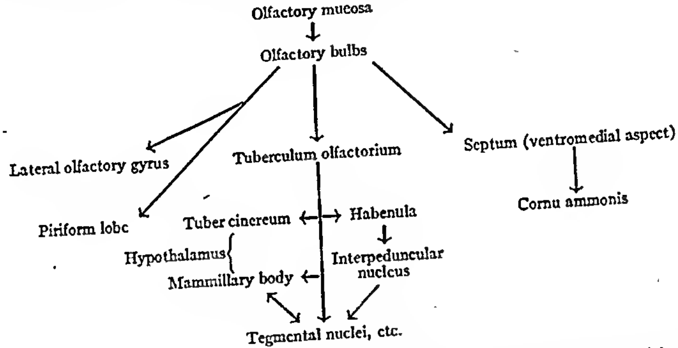
virus for the olfactory neurons in the nasal mucosa. These neurons are in synaptic relation with the mitral cells in the olfactory bulbs, the synaptic junction occurring not directly on the bodies of these neurons but rather at some distance on the dendrites entering the glomeruli. It is significant, therefore, and perhaps indicative of the mode of virus progression that one can find necrosis of the olfactory mucosa with no visible change in the olfactory nerve fibers, glomeruli, and the susceptible cells of the external granular layer which surround them, and yet see almost complete destruction of the mitral cells beyond. In an advanced lesion there is, of course, necrosis of granular cells (they are connected with the mitral cells), particularly of the internal layer, but even then most of the glomeruli and the surrounding cells remain intact.

The axons of the mitral cells grouped in the lateral, intermediate, and medial olfactory striae are said to terminate, either directly or after preliminary synapse in the anterior olfactory nucleus, chiefly among the neurons of the lateral olfactory gyrus, tuberculum olfactorium (region of anterior perforated substance), and questionably in the septum,[4] and it is in these areas also that the further localization of lesions occurs. It is noteworthy that the olfactory bulb lesions are not continuous with those further posteriorly, *i.e.*, that there is between them, almost invariably, an apparently undamaged zone. An analysis of the subsequent localization of lesions (Table II) indicates that the regions involved depend upon the major central connections of olfactory areas which have been previously affected. Thus, in accord with the constant involvement of the intermediate olfactory nucleus (anterior perforated substance, tuberculum olfactorium), one finds always affected the hypothalamus (tuber cinereum and mammillary body), and tegmental nuclei (constituting one of the descending olfactory correlation pathways). Occasionally the pathway through the habenular nuclei and the interpeduncular nucleus is used. In view of the fact that the neurons in the interpeduncular nucleus participating in the olfactory relay are in relation proximally only with cells in the habenular nuclei, it should be noted that when lesions are observed in the interpeduncular nucleus they are invariably also found in the habenular, even though they may be so limited as to require many sections to disclose them. The cornu Ammonis was involved but once and that occurred in a brain which showed necrosis in the ventromedial, septal region anterior to the optic chiasm. The piriform lobes also showed necrosis in only one case and that in association with a lesion of the lateral olfactory gyrus on the same side. Why the tuberculum olfactorium or anterior perforated substance should be so constantly involved, while the lateral olfactory gyrus, piriform, and septal regions

[4] Any simple or short description of the central connections of the olfactory neurons of the second order is necessarily incomplete. The termination of such fibers in the septum of higher mammals is especially open to question. The occasional presence of necrosis in the ventromedial aspect of the septum (parolfactory area, paraterminal body) (see Fig. 6) in some of these mice should, therefore, be interpreted with that in mind.

TABLE II

Scheme Indicating Order of Distribution of Neuronal Lesions after Nasal Instillation of Virus



The purpose of this scheme is to show how the localization of lesions deep within the CNS occurred in accord with the involvement of certain definite, intermediate zones. Thus there were no lesions in the interpeduncular nucleus without involvement of the habenular nuclei and tuberculum olfactorium; none in Ammon's horn without damage to the septum, etc.

are only occasionally affected, is not clear. According to the view of axonal and trans-synaptic progression of the virus, it could perhaps be accounted for if the axons of the greater number of mitral cells were included in it, or if the greater number or the more suitable synaptic connections were in this relay. The diencephalic and tegmental mesencephalic structures which are most frequently affected are the ones which are chiefly concerned in the correlation of olfactory with other impulses and in the function of relay stations on a reflex pathway to the motor and perhaps other nuclei of the hind-brain.

Findings in Old Mice Showing No Clinical Signs of Disease.—Nine mice (eight were approximately 1 year old and one was 31 days old) were studied at various intervals after nasal instillation of virus. Two were sacrificed on the 2nd day, two on the 4th, and one on the 5th, 6th, 7th, 10th, and 14th days, none showing any signs of disease. In none of these animals did the CNS show any changes which could be attributed to the effect of the virus. No definite effect ascribable to the action of the virus on the nasal mucosa was demonstrated. In some of the mice the presence of large numbers of pus cells in the sinuses, tissues, and nasal cavities was perplexing until the same picture was observed in two "normal" old mice which received no virus. No definite conclusion could be reached about the significance of occasional intranuclear, acidophilic bodies.

The absence of obvious lesions in these mice, in spite of the fact that virus can be demonstrated in the rhinencephalon anterior to the optic chiasm between the 2nd and 5th days, is not surprising in view of the fact that they may be practically absent in old mice succumbing with encephalitis after intracerebral injection of the virus.

Findings in Old Mice Showing Signs of Encephalitis or Myelitis.—A varying number of old mice are not resistant to nasal instillation of the virus and develop, after a relatively prolonged incubation period of 8 to 14 days, either signs of encephalitis or merely flaccid paralysis of the posterior extremities. The CNS of two such mice were studied, and the extensive lesions which they exhibited were in marked contrast to the findings in the intracerebrally injected animals. One was sacrificed on the 8th day, when it showed signs of encephalitis for the first time. Lesions quite as extensive as those found in the young mice were present here and in the same regions (see Table I). It should be noted that these extensive lesions clearly due to virus action were present side by side with those of spontaneous encephalitis. The other mouse studied histologically was the only one of a group of eleven 1 year old, similarly instilled mice to develop nervous signs. These appeared in the form of flaccid paralysis of the posterior extremities on the 8th day after nasal instillation, and the mouse was sacrificed 9 days after the onset of paralysis. The striking fact here was that while there was rather marked destruction and neuronophagia in both lateral regions of the anterior horns of the lumbar cord, there was no evident involvement of either the ependyma

and periependymal tissue, the posterior horns of this level, or of any part of the cervical, thoracic, and sacral levels. While insufficient sections of the brain were available to determine the exact tracts by which the cord might have been reached, clearly defined foci of necrosis were found in the hypothalamus, thalamus, and habenular nuclei. It is noteworthy that the spinal ganglia, even of the lumbar level, showed no lesions, and also that there was neither meningeal nor perivascular reaction in the cord.

Pathology after Intraocular Injection

Young Mice.—15 day old mice uniformly succumb after intraocular (vitreous) injection of the virus and are almost as susceptible to inoculation by this route as by the intracerebral. The incubation period is considerably prolonged in 21 day old mice and many of them are completely resistant, even to the highest concentration of virus, while 1 year old animals, with only few exceptions, exhibit no clinical signs of disease whatever. It should also be recalled that while in susceptible mice, 21 days of age or older, it was possible to show by subinoculation methods that the primary spread of the virus was probably along the optic nerve with an early localization in the contralateral diencephalon and mesencephalon, no conclusion could be drawn from the experiments with the 15 day old animals because the virus, when first demonstrated in the CNS, was already widely scattered (2). One of the questions, therefore, was whether the marked and uniform susceptibility of the 15 day old mice is due to a mode of virus progression different from that which obtains among the older animals, or whether the same pathways are pursued in all, the rate of progression being so much greater in the 15 day old mice that the entire nervous system is rapidly invaded by varying amounts of virus.

The pathology after intraocular injection was therefore studied chiefly in 15 day old mice. It was early observed that the CNS lesions in these animals could apparently be accounted for on the basis of primary virus progression only along the decussating optic nerve pathway. In each of three mice, for example, whose right eye was inoculated marked necrotic lesions were present in the left superior colliculus, while the right one appeared entirely uninvolved. To eliminate the possibility that the *left* superior colliculus may be particularly susceptible to the action of this virus and to establish more convincingly that the decussating pathway is used, the brains of two 15 day old mice injected into the left eye were studied. In these two animals the right superior colliculi showed marked necrosis, while the left ones remained uninvolved, thus indicating rather conclusively that in 15 day old mice the virus follows the decussating pathway suggested by the experimental observations on older animals.

A detailed tabulation of the localization of lesions in various regions of the CNS of each of the five mice is presented in Table III. Four were sacrificed 3 days and one 4 days after inoculation at a time when they showed advanced nervous signs or were prostrate. Sections of the inoculated eyes revealed in each instance

TABLE III

Distribution of Necrotic Lesions in the Central Nervous Systems of 15 Day Old Mice Succumbing after Intraocular (Vitreous) Injection of Virus

Region		Eye inoculated and day on which mice were sacrificed				
		Right eye			Left eye	
		3rd	3rd	3rd	4th	3rd
Inoculated eye	Retina	+±	++	++	++++	+
	Cornea, iris, etc.	−	−	−	−	−
Uninoculated eye		−	−	n.s.	−	−
Optic nerve near chiasm	Right	+	n.s.	n.s.	−	−
	Left	−	n.s.	n.s.	+	±
Optic chiasm and tract beyond decussation	Right	−	−	−	+	+
	Left	+	+	+	−	−
Lateral geniculate body	Right	−	−	−	+	−
	Left	+++	−	−	−	−
Medial geniculate bodies		−	−	−	?	−
Superior colliculus	Right	−	−	−	++++	+++
	Left	++++	+++	+++	−	−
Inferior colliculi		−	−	−	−	−
Region of oculomotor and trochlear nuclei		−	−	−	++ (right)	−
Tegmentum	Anterior (including red nuclei)	−	−	−	+++	−
	Posterior (to red nuclei)	?	−	++ (raphé)	+++	± (raphé)
Hypothalamus (contiguous to optic tract)		−	−	±	++ (right side adjacent to optic tract)	−
Thalamus		+ (left lateral nucleus)	−	−	−	−
Mammillary body		−	−	±	+	−
Habenular nuclei		−	−	−	−	−
Interpeduncular nucleus		−	−	−	−	−
Ganglion basale opticum	Right	−	−	−	−	−
	Left	±	±	±	−	−
Cornu Ammonis		−	−	−	−	−
Piriform lobes		−	−	± (left side adjacent to optic tract)	+ (right side adjacent to optic tract)	−
Septum	Right	−	−	−	−	−
	Left	−	−	−	−	−

See legends of Table I.

TABLE III—*Concluded*

Region		Eye inoculated and day on which mice were sacrificed				
		Right eye			Left eye	
		3rd	3rd	3rd	4th	3rd
Tuberculum olfactorium	Right	−	−	−	+	−
	Left	−	−	++	−	−
Lateral olfactory gyrus	Right	−	−	−	−	−
	Left	−	−	+++	−	−
Olfactory bulb	Right	−	−	−	++	−
	Left	−	−	+++	−	−
Corpus striatum		−	−	−	−	−
Neopallial cortex		−	−	−	−	−
Nuclei of pons		±	?	±	++	−
Medulla, reticular substance		−	−	n.s.	+++	++
Cerebellum		−	−	−	−	−
Spinal cord		−	n.s.	−	n.s.	n.s.

a lesion of varying extent in the retina with no evidence of any specific action on any of the other structures. In one mouse (sacrificed on the 4th day) more than one-half of the entire retina was destroyed, leaving not a trace of its original structure or cell outlines. In the other animals the retinae exhibited scattered foci of necrosis involving all the layers to a varying degree. Small numbers of polymorphonuclear and occasional mononuclear leucocytes infiltrated these foci and particularly the outer layers of nerve fibers and ganglion cells. Only a few of the invading cells were found free in the posterior or anterior chambers. The uninjected eyes appeared normal. Sections of eyes removed 4 days after inoculation of broth, or normal mouse brain suspension for control showed only a few polymorphonuclear and mononuclear cells in the posterior and anterior chambers but no changes whatever in the retina. Some inflammatory exudate in the outer coats and muscles of the eye apparently near the site of inoculation was found both after virus and control inoculations.

Examination of the optic nerve of the inoculated eye revealed no change except in the portion near the optic chiasm. This change, consisting of a varying degree of disorganization of the normal structure with necrosis of some of the large interstitial glial cells and infiltration with polymorphonuclear leucocytes, was also found in the middle of the optic chiasm and after the decussation on the side opposite to that of the injected eye. Pathological changes in the optic chiasm beyond the decussation were noted on the left side in mice, which received the virus in the right eye, and on the right in those inoculated in the left eye. In

some of the mice the same sort of change continued in the contralateral optic tract beyond the chiasm.

The one other lesion constantly exhibited by all the mice consisted, as already indicated, of extensive necrosis of the contralateral superior colliculus. In one mouse, sacrificed when prostrate on the 3rd day, this was practically the only finding apart from those already mentioned. Of the three mice which received virus in the right eye, two showed no lesions in the lateral geniculate bodies of either side, while in the third one only the left (*i.e.*, contralateral) lateral geniculate body was affected, showing extensive necrosis of both the dorsal and ventral nuclei with practically no infiltrating inflammatory cells. Here were found many neurons which exhibited the early cytoplasmic and nuclear changes which have already been described. In this mouse the same changes were also present in the left lateral nucleus of the thalamus. Of the two mice, in which the inoculation was made in the left eye, one showed distinct, though not as extensive, necrosis of the right lateral geniculate body with no perceptible involvement of the left one, and in the other there was no lesion in either. It was thus clear that the localization of the most obvious lesions depends on the distribution of the greater number of the axons of the retinal ganglion cells.

Two of the five mice exhibited significant unilateral lesions in the olfactory pathway. The chief interest of this involvement lies in the fact that in both cases it was the contralateral olfactory pathway which was affected. In one of these mice, whose right eye was inoculated, the right olfactory pathway appeared entirely normal, while moderately extensive foci of necrosis (not contiguous with one another) were present in the tuberculum olfactorium, lateral olfactory gyrus, and olfactory bulb of the left side. In the other mouse the virus was injected in the left eye, and the left olfactory pathway appeared normal, while the tuberculum olfactorium and olfactory bulb of the right side showed distinct foci of necrosis. It is apparent from this localization that these lesions cannot be explained on the basis of an escape of some of the virus from the conjunctival sac into the nose, for then they should have been on the same side as the inoculated eye. The explanation for the involvement of the contralateral olfactory pathway may, perhaps, be found in the changes spreading from the optic tract, beyond the decussation of the optic chiasm, to the structures with which it is intimately connected by contiguity, *i.e.*, the tuber cinereum, the ganglion basale opticum (an olfactory ganglion), and the piriform lobe. Varying numbers of necrotic cells were found in all these structures in the zones which were in contact with the necrotic portion of the optic chiasm and tract. These changes were least noticeable in the mice sacrificed on the 3rd day, but quite marked in the one killed on the 4th day. It is apparent, however, that the further distribution of lesions from these olfactory centers anteriorly or posteriorly is not by contiguity, but apparently axonal and trans-synaptic from one level or station to the next. The two mice just described also showed necrosis of a small number of cells in the mammillary body and extensive destruction of the ventral portion of the posterior tegmentum (*i.e.*, posterior to the level of the red nuclei).

In none of the four mice sacrificed on the 3rd day were there any perceptible lesions just ventral to the aqueduct of Sylvius (region of nuclei of third and fourth cranial nerves and Edinger-Westphal nucleus), nor in any other part of the anterior tegmentum, (*i.e.*, portion containing red nuclei). The mouse sacrificed on the 4th day (left eye injected) showed marked necrosis in the region of the nuclei of the 3rd and 4th cranial nerves on the right side and almost complete destruction of the anterior and posterior regions of the tegmentum. The pons showed a varying number of necrotic cells in three mice, and the medulla some foci in the reticular substance of two.

No lesions were found in any of the mice in the cornu Ammonis, corpus striatum, neopallium, cerebellum, and spinal cord. It should also be noted that no lesions were seen in the ciliary, Gasserian, submaxillary, and superior cervical sympathetic ganglia.

Old Mice.—It has already been shown that in old mice which are resistant to intraocular injection, no virus can be demonstrated in the CNS (2). The chief interest, therefore, was in determining the effect of the virus in the eye, where its progression is apparently held up. The eyes of two old mice sacrificed on the 4th day showed no visible lesions in the retina nor any more cellular infiltration than was found in eyes injected with normal mouse brain suspension for control. The optic nerves and CNS similarly revealed no change.

Pathology after Intramuscular Injection

Young and Old Mice.—Young mice receiving virus in the muscles of one leg develop flaccid paralysis, first of the inoculated extremity, and succumb with signs of an ascending myelitis. Old mice, on the other hand, exhibit no signs of disease after intramuscular injection of the largest amounts of virus (10^7 M.C.L.D.). In the young mice the virus has been shown to multiply at the site of inoculation and to invade the spinal cord by way of the peripheral nerves, while in old mice neither local multiplication nor invasion of the peripheral nerves or CNS was demonstrable (2). One of the questions which arose during these experiments was whether the local multiplication of virus in the young mice occurred in nervous structures or in the muscle and other non-nervous elements. Another question was whether the intramuscular injection of large amounts of virus in old mice had any effect on the muscle or nervous tissue which might be demonstrable histologically though not by animal passage.

To answer these questions, histological studies were made of the muscles and peripheral nerves of the inoculated legs of young and old mice at various intervals after injection. For control, young and old mice were given similar amounts of normal mouse brain suspension and the above mentioned tissues removed at the same intervals after inoculation. To aid in the localization of the site of inoculation, a small amount of powdered charcoal was added both to the virus and normal brain suspensions. In mice sacrificed on the 2nd day the reaction to the normal mouse brain-charcoal powder mixture consisted of an infiltration of the interstitial connective tissue with polymorphonuclear and mononuclear cells. The

number of the former cells diminished on the 3rd day, and on the 5th, day the inflammatory exudate consisted almost entirely of mononuclear cells. The reaction was practically the same in both the young and old mice. There was no evidence of any involvement of the muscle or nerve tissue at the injected site, and sections of the sciatic nerves appeared normal.

In the old mice injected with virus there was no perceptible difference from the reaction to normal tissue just described, while in the young mice, on the other hand, there appeared definite evidence of a direct attack of the virus on the muscle tissue. At 2 days after injection (the period when considerable multiplication of the virus was demonstrated and successful muscle to muscle passage was carried out (2)), there was as yet no evident necrosis of the muscle fibers and no appreciable difference in the inflammatory reaction from that observed in the control animals. On the 3rd day, fragmentation and hyaline-like necrosis of a small number of muscle fibers became apparent and an increase in the inflammatory exudate consisting mostly of polymorphonuclear leucocytes, many of which appeared to be phagocytizing the fragmented muscle fibers. On the 5th day practically the same picture was seen but in an exaggerated form: A large number of muscle fibers was completely necrotic and the phagocytosis of muscle fragments by numerous polymorphonuclear leucocytes (which may perhaps be defined as myophagia) closely resembled the picture of neuronophagia in the nervous system. Hypertrophy and proliferation of the sarcolemmal nuclei formed another prominent feature of the reaction at this time. No inclusions were seen in any of the cells on the 2nd, 3rd, or 5th days and no evidence could be found of a direct attack of the virus on the interstitial connective tissue, blood vessels, or nerve trunks lying in the vicinity of necrotic muscle tissue. Longitudinal sections through the sciatic nerves of the inoculated legs showed no inflammatory reaction at any time nor any other perceptible abnormality on the 2nd and 3rd days; on the 5th day the appearance was suspicious of a fragmentation of a certain number of nerve fibers, but no definite conclusion is possible with the method employed.

These histological findings thus proved to be in accord with the experimental results (2) and further elucidated the nature of local virus multiplication at the site of intramuscular injection in the young mice.

The CNS of two mice (15 days old) injected with virus into the right leg were examined; one on the 4th day, when it exhibited only paralysis of the posterior extremities, and the other on the 5th day immediately after death. In the animal sacrificed on the 4th day the outstanding lesion was found in the lower lumbar cord, where it seemed to be confined to the neurons of the anterior horns. These cells were in various stages of necrosis and early neuronophagia was present. A few of the blood vessels were surrounded by one or two layers of mononuclear and polymorphonuclear cells. The meninges showed no evidence of virus attack. The spinal and sympathetic ganglia at the lumbar levels of the cord having considerable anterior horn cell involvement exhibited neither neuronal necrosis nor inflammatory reaction. It would appear that either the virus entered the cord entirely by the efferent axons of the anterior horn cells distributed to the muscles

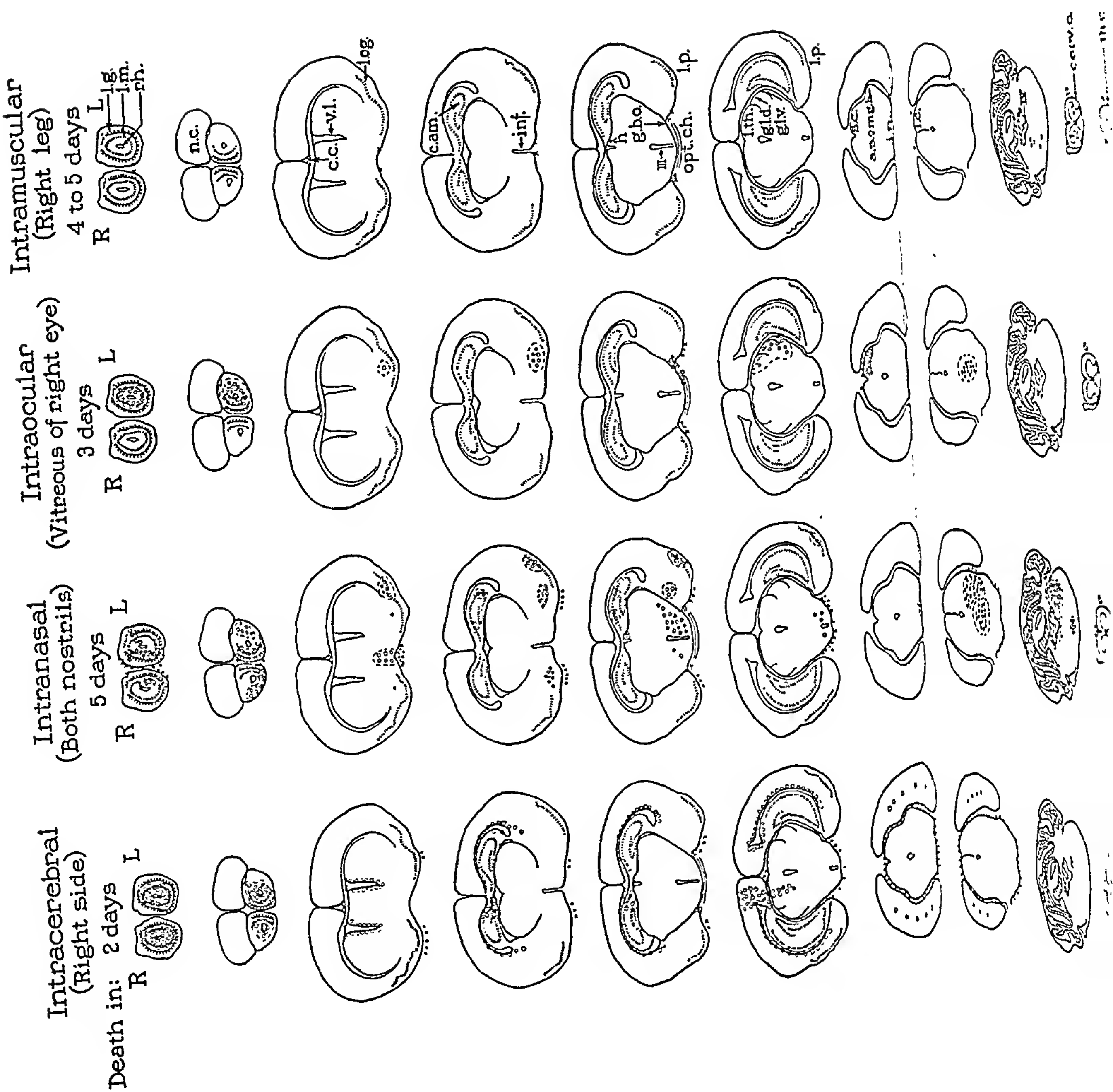
which it attacked, or if it also pursued the afferent sensory pathway, the neurons in the spinal ganglia must undergo necrosis much less readily than the motor cells. The thoracic and cervical levels of the spinal cord showed little change with the possible exception of some doubtful intranuclear inclusions in a few neurons. The only other neuronal lesions in the CNS were found in the ventral aspect of the medulla, slightly cephalad to the decussation of the pyramidal tracts, and particularly on the right side in the region of the lateral reticular and possibly of the olivary nuclei. Here there was evidence of early nerve cell necrosis, and some interstitial and perivascular infiltration with polymorphonuclear and mononuclear cells. A small number of these cells could be seen in the meninges of the ventral aspect of the brain as far cephalad as the olfactory bulbs.

In the second animal in which the infection was allowed to go on to a fatal termination, there was complete necrosis of almost all neurons, both in the anterior and posterior horns of the lumbar cord with very little inflammatory reaction. Thus neuronal destruction had already spread to most of the sacral and thoracic portions of the spinal cord, while in the cervical portion necrosis was still in its earlier stages. The only other nervous lesions noted were again in the reticular substance of the medulla, where foci of necrosis and neuronophagia could be found. The spinal ganglia appeared entirely uninvolved.

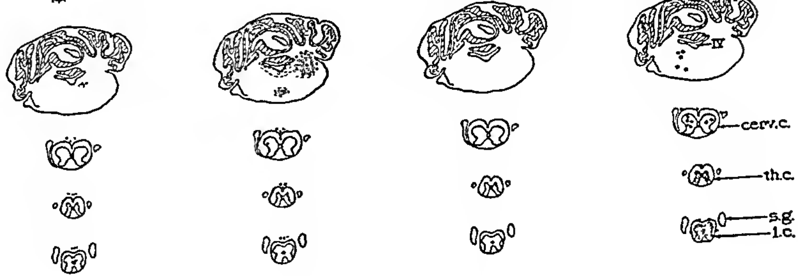
In summary it may be pointed out that the CNS lesions, after inoculation of the virus into the muscles of one leg, are distinctly different in distribution from those which follow intracerebral, intranasal, or intraocular injection of the virus. Their localization is in accord with a primary, insulated, axonal transmission of the virus, the most evident damage being observed not along the course of the axons but rather at the site of their cell bodies. The absence of lesions in the spinal ganglia, even at death, strongly suggests that the invasion of the virus into the spinal cord may have occurred chiefly along the efferent fibers supplying the affected muscles.

Correlation between Distribution of Lesions and Presence of Virus in the Central Nervous System

One is impressed with the fact that many areas in the CNS which were shown to contain virus by animal passage (2) exhibited no evident histological changes. This is particularly apparent among the 15 day old animals which were injected intraocularly. Thus, while animal passage revealed virus among other zones in the homolateral diencephalon and mesencephalon, and in the occipital cortex at an early stage, no lesions were found in these regions in any of the mice. In general, the impression is gained that in the case of vesicular stomatitis, virus multiplication precedes recognizable cytological changes by a day or two and that lesions appear where the virus is delivered first and perhaps in largest amount,



Intramuscular (Right leg) 4 to 5 days
Intraocular (Vitreous of right eye) 3 days
Intranasal (Both nostrils) 5 days
Intracerebral (Right side) 2 days
Death in:
R
L
l.g.
l.m.
r.h.
n.c.
c.c.
v.l.
l.og.
c.am.
inf.
h
g.b.o.
III
opt.ch.
l.p.
l.th.
gl.d.
gl.v.



:: — Indicates location of cellular necrosis.
xx — " meningeal reaction.
◯ — Broken line around a focus indicates that the lesion was not present in all the mice examined.

TEXT-FIG. 1. Influence of route of inoculation on CNS pathology in young mice (vesicular stomatitis virus, N. J.). Diagrammatic representation of localization of lesions in CNS of young mice after different routes of inoculation. The outlines of the cross sections of the hrain and cord are about 4 times actual size. The lesions found in several mice inoculated hy the same route were combined on one series of cross sections.

R, right side; L, left side; a.s., aqueduct of Sylvius; c. am., cornu Ammonis; c.c., corpus callosum; cerv. c., ccrvical cord; g.b.o., ganglion basale opticum (preoptic nucleus); g.l.d., dorsal nucleus of lateral geniculate body; g.l.v., ventral nucleus of lateral geniculate hody; h, hahenula; i.c., inferior colliculus; inf., infundihulum; l.c., lumbosacral cord; l.g., lamina glomerulosa of olfactory bulh; l.m., layer of mitral cells; l.o.g., lateral olfactory gyrus; l.p., lobus piriformis; l.th., lateral nucleus of thalamus; m.g.b., medial geniculate body; n.c., neopallial cortex; opt. ch., optic chiasm; rh., rhinocele (anterior extension of lateral ventricle); s.c., superior colliculus; s.g., spinal ganglion; th.c., thoracic cord; v.l., lateral ventricle; III. third ventricle; IV, fourth ventricle; i. n., interpeduncular nucleus.

death terminating the disease before the other areas, infected later and perhaps less heavily, have developed demonstrable changes. It does not appear that in young mice certain areas are more resistant to necrosis than others, because by varying the route of inoculation almost all regions of the CNS can be shown to be susceptible. Considering the extensive connections between various regions of the CNS, it is easy to understand how virus can spread almost simultaneously to many remote parts of the brain, but the localization of lesions chiefly in the zones which receive the greatest number of axons from neurons primarily infected is in good agreement with the hypothesis of axonal and trans-synaptic progression of the virus. In intracerebrally injected old animals, on the other hand, animal passage clearly demonstrated that virus had multiplied in widely scattered areas of the CNS within 24 hours after injection (1) and yet in moribund mice sacrificed on the 3rd and 4th days recognizable lesions were either entirely absent or involved only a small number of isolated cells. This observation indicates that at least in some of the older animals the cells have become distinctly more resistant to necrosis resulting from virus multiplication. It is to be recalled, however, that while the cells retain their essential structure and show no other changes recognizable by the methods employed, there is, nevertheless, impairment of their function as evidenced by the clinical signs of encephalitis and death of the animal. Further study may reveal that the resistance of nerve cells of old mice to necrosis may not be limited to vesicular stomatitis virus. In describing the pathology of yellow fever encephalitis in two mice, one 6 days old and one adult, Goodpasture (17) noted that in the young mouse there was a great deal of neuronal necrosis with almost imperceptible inflammatory reaction, while in the adult mouse despite the abundant perivascular and diffuse cellular exudation, neuronic alterations were difficult to find. While the inflammatory reaction in the old animal may have occurred in response to imperceptible virus action on the cells, it resembles very closely the picture of spontaneous encephalitis.

SUMMARY AND DISCUSSION

It will be well to restate the main problem at this point and to examine how far the accumulated data can help to elucidate it. The problem is this: Why are old mice generally resistant to all forms of peripheral inoculation of vesicular stomatitis virus when intracerebral injection is equally fatal for mice of all ages? The results of experiments in which the presence of virus was demonstrated by animal passage suggested that the reason can perhaps be found in (*a*) the different mechanisms of virus progression after intracerebral and peripheral injection, and (*b*) the development with age of localized barriers capable of halting the spread of virus (1, 2). The present study sought histological evidence for the nature of virus progression

and for the changes observed in the older animals. The results clearly demonstrate that after intracerebral injection virus spreads along an open system, the lesions being distributed almost entirely in contiguity with the ventricles and their extensions, while after peripheral inoculations the evidence points to progression of the virus in a closed system of neurons and their processes, at least in the stage preceding neuronal necrosis, the distribution of lesions depending upon the central connections of the primary neurons connected with the inoculated site. Thus, in young mice, nasal instillation of the virus was followed by necrosis of a long chain of neurons, starting with those in the olfactory mucosa and progressing through specific zones of the olfactory pathway, pursuing the same order in which the various regions are known to have their major connections with one another. It is important to note that after nasal instillation the apparent lesions were present where the cell bodies of the neurons are situated, and not along the tracts connecting one group of neurons with another, which accounts for the lack of contiguity between the affected zones and the normal appearing, intervening areas. The assumption that the primary progression of the virus in this case occurs in a closed system is based on the absence of lesions in unrelated areas contiguous to those which are necrotic and to the tracts which connect one affected zone with another.

Additional evidence for the assumption that the initial dissemination of peripherally injected virus is in a closed system is found in the decussating optic nerve pathway primarily pursued by the intraocularly injected virus. The progression of the virus along this decussating pathway was indicated in the experimental data obtained on mice 21 days or older, while in younger animals the spread of virus was so rapid and diffuse that the pathways along which it might have occurred remained obscure (2). In the present study, in which 15 day old mice were used, the lesions in the retinal neurons and the constant involvement of only the contralateral superior colliculus left little doubt that the *primary* spread of the virus, even in these very young animals, must have occurred within the retinal neuron processes (axons) which decussate in the optic chiasm (in the mouse, as in the rat, very few of these go to the homolateral side) and synapse chiefly with the neurons of the contralateral superior colliculus and

also, apparently to a lesser extent, with those of the contralateral external geniculate body, where lesions were also demonstrated. Virus spreading in the optic nerve along the perineural subarachnoid space would be found at the base of the brain at the optic chiasm; virus extending along the interstitial spaces in the optic nerve should involve not only the nuclei of both sides of the optic pathway but also non-optic structures, such as the medial geniculate bodies, posterior colliculi, etc., by means of the commissures of von Gudden and of Meynert, whose fibers course through the chiasm. The highly specific localization observed in the present study is best accounted for by progression along the suggested closed pathway. Hurst (10) observed that poliomyelitis virus, after injection into the left sciatic nerve, may, after invading the lumbar cord, be found first in the contralateral motor cortex or thalamus and he suggested that this was evidence of progression along a decussating pathway and in favor of the axonal hypothesis of virus spread. It was not shown, however, that this particular localization was specifically related to the introduction of virus in the left sciatic nerve, or that it could be reversed by inoculating the sciatic nerve of the opposite side. The hypothesis proposed by Hurst, however, finds support in the present instance for (*a*) the superior colliculi never showed lesions after intracerebral, intranasal, or intramuscular inoculations, and (*b*) necrosis was produced in either the right or the left superior colliculus, depending on whether the virus was injected into the left or right eyes.

The localization of lesions after injection of virus into the muscles of one leg indicated that in the young the invasion occurred along the local peripheral nerves, especially the motor fibers (neurons destroyed in the lumbar cord with those in the spinal ganglia intact), after a primary attack on the muscle itself. The only other lesions found at a late stage were in the reticular substance of the medulla, the olfactory portions of the brain appearing entirely normal. In this respect the mechanism of progression of intramuscularly injected vesicular stomatitis virus differs from that of eastern equine encephalomyelitis and pseudorabies viruses similarly injected into mice of the same age and breed: the former (E.E.E.) invades the central nervous system in the majority of instances, by being eliminated on the nasal mucosa and then along the olfactory pathways (18), while the latter

appears to employ chiefly the local sensory fibers, attacking primarily the neurons in the spinal ganglia (unpublished observations).

Because the CNS of old mice remain for the most part susceptible to vesicular stomatitis virus (although definite evidence of resistance to necrosis of the neurons was observed), and because after intracerebral injection the virus has been shown to spread in an open (ventricular) system, it is clear why young and old mice are equally susceptible to inoculation by this route. After peripheral inoculation, however, it has been amply demonstrated by experimental and histological methods that the spread of this virus begins and continues, at least until the cells disintegrate, in a closed system within the neurons and their processes and apparently also across the synapses. The halting of the virus somewhere in the anterior rhinencephalon after nasal instillation in resistant mice (1) would appear to be due to an arrest in an insusceptible neuron or an impenetrable synapse somewhere in the chain, and to the failure of the affected neurons to disintegrate (no lesions were found in the CNS of these mice) and thus to liberate the virus into the open system. After intramuscular injection, on the other hand, the virus encounters a different kind of muscle cell in the old mouse, and its inability to invade the nerves may perhaps be bound up with its demonstrated inability to attack and multiply in these changed muscle cells, although the rôle of a possible alteration in the terminal nerve endings themselves is not yet clear. After intraocular injection, the virus fails to affect visibly the retinal neurons of resistant old mice and the further invasion of the CNS is inhibited. The resistance of old mice to peripheral inoculations of vesicular stomatitis virus thus appears to be the result of (*a*) changes produced by age not in the whole animal but in certain specific, isolated structures, and (*b*) the special mode of progression of peripherally injected virus.

It may be of interest to point out two phenomena which may perhaps be related to the one investigated in the present study. Tobacco mosaic virus has been found to produce different types of disease in certain plants of different ages; thus a widespread, systemic necrosis leads to the death of young *Nicotiana rustica* plants, while in old plants it is possible to produce necrotic foci in many parts of the plant by direct inoculation, although generalization does not

EXPLANATION OF PLATES

The sections in the plates are of material obtained from 15 day old mice and stained with phloxine and methylene blue.

Plate 6

Fig. 1. 4 days after injection of virus into vitreous humor of left eye. A, uninoculated eye; note normal appearance of retina. B, inoculated eye; arrows point to necrosis of retina. × 28.

Fig. 2. Necrosis of *right* superior colliculus after inoculation of *left* eye. × 31.

Photographed by Joseph B. Haulenbeck and Louis Schmidt

Plate 7

Fig. 3. Necrosis of *left* superior colliculus after inoculation of *right* eye. × 31.

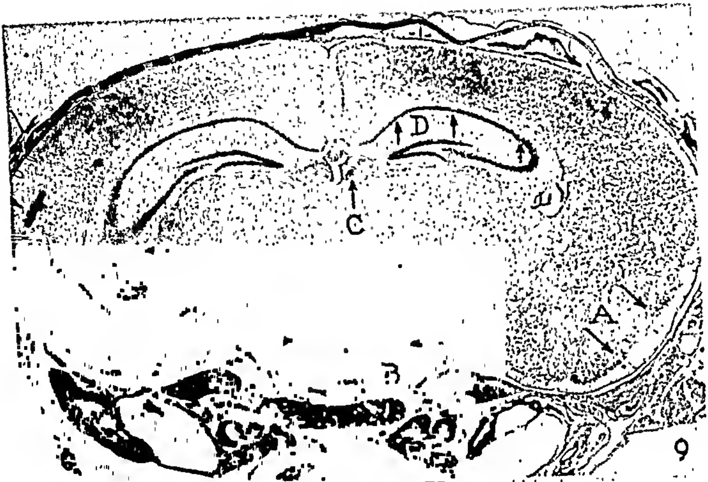
Fig. 4. 4 days after nasal instillation of virus. Note necrosis and desquamation of part of olfactory mucosa. Left olfactory bulb shows extensive necrosis in all layers except the outermost one of nerve fibers; arrows point to almost complete disappearance of mitral cells. The right olfactory bulb shows a much earlier lesion, involving only a small number of the mitral cells and neurons in the lamina gelatinosa; note the normal appearance of the three outer layers: nerve fibers, glomeruli, and external granular layer. For normal architecture of olfactory bulbs, see also Fig. 1. × 35.

Photographed by Joseph B. Haulenbeek and Louis Schmidt

PLATE 9

FIG. 9. 4 days after nasal instillation. Arrows point to necrosis in piriform lobe at A, hypothalamus, B, habenular nucleus, C, and cornu Ammonis, D. × 15.

FIG. 10. Intracerebral inoculation. Arrows point to necrosis of portions of cornu Ammonis and neopallial cortex forming the walls of the lateral ventricles. × 15.



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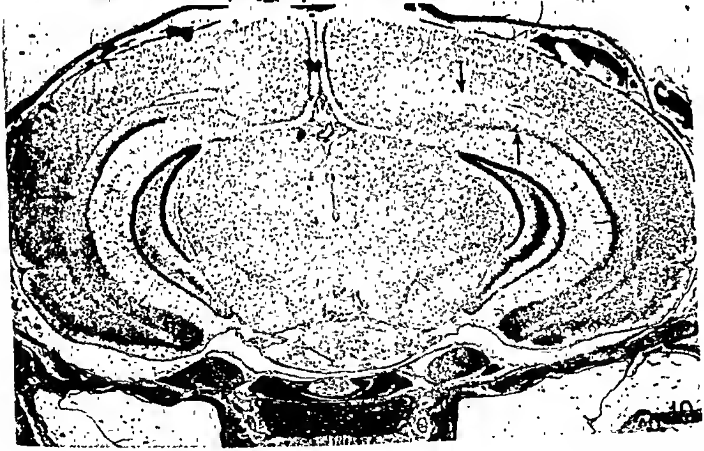

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Photographed by Joseph B. Haulenbeek and Louis Schmidt

INFLUENCE OF HOST FACTORS ON NEUROINVASIVENESS OF VESICULAR STOMATITIS VIRUS

IV. Variations in Neuroinvasiveness in Different Species*

By ALBERT B. SABIN, M.D., and PETER K. OLITSKY, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

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The preceding investigations have thrown some light on the cause of variations in the capacity of vesicular stomatitis virus to give rise to encephalitis or myelitis in young and old animals of the same species (mice) (1). This virus, like a number of others, displays also distinct variations in its ability to cause disease of the central nervous system (CNS) in animals of different species under conditions of spontaneous infection or experimental peripheral inoculation. In nature it produces vesicular lesions in horses and cattle but is not known to involve the CNS. In guinea pigs suitable peripheral inoculation also gives rise to vesicular lesions unaccompanied by CNS disease, while in mice of proper age such injections are almost always followed by encephalitis or myelitis, depending upon the site of peripheral inoculation. The present investigation was undertaken to determine, for a single virus, the host factors which are responsible for the protection of the CNS from manifest disease in one species under conditions in which it is constantly involved in animals of another species.

Methods

The technique employed in tracing the progression of the virus in the nervous system and other parts of the body from various sites of inoculation was essentially the same as those already described in the similar studies on mice (1). Necessary variations will be described in the text. The guinea pigs used in the present inves-

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* Presented before the American Association of Immunologists and the American Association of Pathologists and Bacteriologists, April 9, 1936, (*Am. J. Path.*, 1936, 12, 738.)

tigation were of mixed, unknown breed, and the exact ages were known only for the younger animals. Both the New Jersey and Indiana mouse passage strains were employed and tests for virus in guinea pig tissues were made by the intracerebral injection of suitable suspensions in 21 to 30 day old Rockefeller Institute albino mice.

EXPERIMENTAL

Comparative Infectivity of the Virus for Mice and Young and Old Guinea Pigs by the Intracerebral Route.—It is known that guinea pigs develop encephalomyelitis after intracerebral injection of vesicular stomatitis virus (2), but it has not been ascertained whether the minimal amount required to induce manifest CNS disease in them is greater or smaller than that in mice. The purpose of the following experiments, therefore, was to determine in simultaneous tests the comparative susceptibility of the CNS of mice and guinea pigs when the virus was introduced directly into the brain. This information was necessary to indicate whether the absence of manifest CNS disease after peripheral inoculation in guinea pigs was the result of a lesser susceptibility of nervous tissue in general, or of factors related to the mode of virus progression from peripheral sites.

Tests were performed with the Indiana (Ind.) and New Jersey (N. J.) strains, using fresh centrifuged mouse brain suspensions in dilutions from 10^{-1} to 10^{-7} for intracerebral injection into 3 week old mice, young guinea pigs, 8 to 12 days old, and adult guinea pigs 2 months or more of age. The volume injected was 0.03 cc. in each mouse and 0.15 cc. for each guinea pig. One of the difficulties encountered in these tests was due to the fact that broth,[1] which is used as routine for preparing and diluting the virus suspensions, proved to be highly toxic upon intracerebral injection in guinea pigs, 2 months of age or older, more than 50 per cent of the animals dying within less than 12 hours; young and old mice and the young guinea pigs showed no such effect. Although with physiological salt solution as the suspending medium the titer of the virus is almost always tenfold less than with broth, it, nevertheless, had to be used in the comparative intracerebral tests. 110 guinea pigs and forty-eight mice were required to obtain the data necessary for establishing the relative susceptibilities of the CNS of the two species.

The results of three series of tests for each strain of the virus are shown in Table I. The most significant fact for the present investi-

[1] This broth is the kind usually called hormone broth containing chiefly beef heart infusion and 1 per cent peptone.

gation is that the CNS of young guinea pigs and young mice proved to be equally susceptible to both the N. J. and Ind. strains when the virus was injected directly into the brain, *i.e.* the minimal cerebral lethal dose (M.C.L.D.) for mice also caused a clinically apparent CNS disease in young guinea pigs. At the same time two other facts emerge: (*a*) while young and old mice are equally susceptible to intracerebral injection, old guinea pigs (2 months or older) seem to require on an average about 10 times as much virus as young ones (8 to 12 days) for the production of manifest CNS disease; and (*b*) primary flaccid paralysis of the posterior extremities occurred in twenty-five of twenty-seven guinea pigs which succumbed with the Indiana strain and in only two of thirty-three with the N. J. strain. Only about half the number of guinea pigs paralyzed with the Indiana strain died and the others recovered either completely or more often with marked residual paralysis. Guinea pigs succumbing with the N. J. strain exhibited varied encephalitic signs and all died. With respect to the development of flaccid paralysis of the extremities as the primary and chief nervous sign following intracerebral inoculation, the Indiana strain in guinea pigs thus closely resembles poliomyelitis in *rhesus* monkeys.

Comparative Infectivity of the Virus for Young Mice and Young and Old Guinea Pigs by the Nasal Route.—Since there was no record of the effect of nasal instillation of vesicular stomatitis virus in guinea pigs, the present experiment was undertaken to determine it simultaneously in young mice and young and old guinea pigs with a virus suspension whose infectivity by intracerebral inoculation would be established at the same time.

The tests recorded in Table II were carried out simultaneously with those described under Experiment A in Table I, using the same suspensions of virus in broth. 0.075 cc. was instilled in each nostril, using a number of guinea pigs for each of the 10^{-1}, 10^{-2}, and 10^{-3} dilutions. The largest number of guinea pigs, 12 (7 to 9 days old) and 12 (approximately 90 days old), were given the 10^{-2} dilution of the N. J. virus. 15 day old mice were used for control and each received 0.03 cc. of the indicated dilutions.

As was to be expected, all the mice developed encephalitis and died. None of the twelve young and old guinea pigs instilled with the Indiana virus, however, nor any of the fourteen adult guinea pigs

TABLE I

Comparative Infectivity of Vesicular Stomatitis Viruses for Mice and Guinea Pigs by the Intracerebral Route

Strain of vesicular stomatitis virus	Experiment	Animals used	Age	Dilution of virus						
				10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Indiana	A Virus in broth	Guinea pigs	8–12 days	n.t.	n.t.	PP4S* PP4D12†	PP5S PP5S	PP8S PP5D21	PP6S PP7S	n.t.
			Over 2 mos.	"	"	D+ 0	D+ 0	D+ D+	0	"
		Mice	21 days	"	"	n.t.	n.t.	3, 3, 4	4, 4, 0‡	"
	B Virus in saline	Guinea pigs	10 days	n.t.	n.t.	n.t.	PP5S 0	E18D22 0	0 0	0 0
			About 10 wks.	E9D10 PP5S PP5R10§ 0	PP3D5 PP5D10 PP5D10 PP5D28	PP6D8 PP6S PP7R11 0	PP6S PP6D11 D6 0	0 0	n.t.	n.t.
		Mice	21 days	n.t.	n.t.	n.t.	n.t.	4, 4, 0	0, 0, 0	0, 0, 0
	C Virus in saline	Guinea pigs	10 days	n.t.	n.t.	n.t.	PP5S 0	0 0	0 0	n.t.
			About 3 mos.	"	"	PP3D5 PP7D16	PP5D7 0	0 0	n.t.	"
		Mice	21 days	"	"	n.t.	n.t.	4, 5, 0	0, 0, 0	0, 0, 0

New Jersey	A Virus in broth	Guinea pigs	10 days	n.t.	n.t.	n.t.	E4D6‖ E6D9	D+ E6D11	D4 E6D8	n.t.
			About 3 mos.	D+ D+	E3D4 E4D6	D+ PP3D4	D+ PP5D8	D+ 0	0 0	"
		Mice	21 days	n.t.	n.t.	n.t.	n.t.	3, 3, 4	3, 4, 5	"
	B Virus in saline	Guinea pigs	10 days	n.t.	n.t.	n.t.	D2 E3D3	E3D8 E7D9	D4 E4D7	E8D13 0
			About 3 mos.	"	E3D4 E3D5 E4D6	E3D5 E3D5 E4D5 E7D11	E4D6 E6D10 E7D10 E11D13	E7D10 E7D10	D7 0	n.t.
		Mice	21 days	"	n.t.	n.t.	n.t.	3, 4, 4	3, 3, 0	0, 0, 0
	C Virus in saline	Guinea pigs	10 days	n.t.	n.t.	n.t.	n.t.	E4D5 E4D7	0 0	0 0
			Over 3 mos.	"	"	"	E4D7 0	0 0	0 0	n.t.
		Mice	21 days	"	"	"	n.t.	4, 5, 0	0, 0, 0	0, 0, 0

0 = inoculated animal remained well.
n.t. = not tested.
D+ = died within less than 12 hours: broth toxicity.
* PP4S = flaccid paralysis of posterior extremities 4 days after inoculation; survived.
† PP4D12 = flaccid paralysis of posterior extremities 4 days after inoculation; died 12th day.
‡ 4, 4, 0 = three mice inoculated of which two died on the 4th day and one survived.
§ PP5R10 = flaccid paralysis of posterior extremities 5 days after inoculation; complete recovery on 10th day.
‖E4D6 = signs of encephalitis 4th day and died 6th day.

which received the N. J. strain showed any signs of disease. One of the twelve 7 to 9 day old guinea pigs instilled with the 1:100 dilution of N. J. virus developed distinct encephalitic signs (coarse tremors, incoordination, etc.) on the 5th day which lasted for only 3 to 4 days, the animal making a complete recovery. Further experience with nasal instillation of the N. J. strain indicates that guinea pigs over 2 months of age show no signs of disease whatever, while no more and probably less than one of about twenty of the very young ones exhibits some transitory encephalitic signs. When the results for the

TABLE II

Comparative Infectivity of Vesicular Stomatitis Viruses for Guinea Pigs and Mice by the Nasal Route

Strain of virus	Animals used	Age	Dilution of virus		
			1:10	1:100	1:1000
Indiana	Guinea pigs	8–12 days	0, 0	0, 0	0, 0
		Over 2 mos.	0, 0	0, 0	0, 0
	Mice	15 days	4, 4, 4	4, 5, 5	5, 6, 6
New Jersey	Guinea pigs	7–9 days	0, E?D6	0, 0, 0, 0, 0, 0 0, 0, 0, 0, 0, E5 Rec.	n.t.
		About 3 mos.	0, 0	0, 0, 0, 0, 0, 0 0, 0, 0, 0, 0, 0	n.t.
	Mice	15 days	4, 4, 4	4, 4, 5	n.t.

E?D6 = dubious signs of encephalitis; died 6th day.
E5 Rec. = signs of encephalitis appeared on 5th day; complete recovery.
Other legends as in Table I.

young animals of the two species are compared it appears that while both are equally susceptible to intracerebral inoculation, nasal instillation of the virus (in adequate amounts) constantly leads to encephalitis in young mice and, with only rare exceptions, not in the young guinea pigs. This in itself suggested that the absence of manifest CNS disease after peripheral inoculation in guinea pigs was not the result of a lower susceptibility of the entire nervous system, but was influenced rather by factors which modified the progression of nasally instilled virus in this species.

Spread of the Virus (N. J. Strain) into Central Nervous Systems of Young and Old Guinea Pigs after Nasal Instillation.—It will be recalled that in old mice, remaining entirely well after nasal instillation of the virus, the CNS was, nevertheless, invaded along the olfactory pathway and that the progression of the virus appeared to be arrested somewhere in the anterior rhinencephalon (1). The object of the following experiment was to disclose whether or not the resistance of guinea pigs was brought about by a similar mechanism.

TABLE III

Spread of Vesicular Stomatitis Virus (N. J. Strain) into Central Nervous System of Young and Adult Guinea Pigs after Nasal Instillation

Experiment	Time after nasal instillation of virus 0.5×10^5 M.C.L.D.	Presence of virus in					
		Young (10–12 days)			Adult (10–12 wks.)		
		Blood	Anterior rhinenceph-alon	Rest of brain	Blood	Anterior rhinenceph-alon	Rest of brain
	days						
A	2	0, 0, 0	3, 3, 0*	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
	4	0, 0, 0	3, 6, 0	3, 3, 3	0, 0, 0	0, 0, 0	0, 0, 0
	8	0, 0, 0	4, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
B	2	0, 0, 0	2, 3, 4	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
	4	0, 0, 0	2, 2, 6	3, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
	8	0, 0, 0	3, 3, 3	3, 3, 3	0, 0, 0	0, 0, 0	0, 0, 0
C	6	n.t.	n.t.	n.t.	0, 0, 0	0, 0, 0	0, 0, 0
	10	"	"	"	0, 0, 0	0, 0, 0	0, 0, 0

* 3, 3, 0 = material injected intracerebrally in three mice of which two died on the 3rd day and one remained well.

Groups of guinea pigs, 10 to 12 days, and 10 to 12 weeks of age, were given nasal instillations of approximately 50,000 M.C.L.D. of the N. J. virus. At the intervals indicated in Table III, different animals were sacrificed and their blood and parts of the brain were tested for virus by intracerebral inoculation in mice. For this series of tests the brain was divided, as in mice, into two parts: the olfactory bulbs and structures ventral to the rhinal fissure up to the optic chiasm constituted one part, referred to as the anterior rhinencephalon, and a number of pieces from representative regions of the rest of the brain constituted the other. The former was ground up in 3 cc. of broth and the latter in 6 cc. of which 0.03 cc. was injected into each of three mice. Great care was taken to establish (by

examination of film preparation, culture, and passage) that mice which succumbed did so as a result of infection with vesicular stomatitis virus; it was in the course of such tests that toxoplasma were isolated on one occasion (3).

The results shown in Table III present a different picture from that obtained in mice. In the adult guinea pigs no virus was detected at any time between the 2nd and 10th days in either the blood or any part of the brain. In the young guinea pigs, on the other hand, virus was regularly found in the brain with none in the blood. In the animals sacrificed on the 2nd day virus was detected only in the anterior rhinencephalon, but in those killed on the 4th and 8th days after nasal instillation it was present there and also in the remainder of the brain. While the fact that virus was detectable only in the anterior rhinencephalon on the 2nd day is to some extent evidence against the widespread dissemination expected of spread in an "open system," it was, nevertheless, clear that it subsequently involved other parts of the brain and was not arrested in the same manner or site as in the resistant mice. It was, therefore, necessary to determine whether this later spread of the virus was diffuse and without relation to the tract connections of the olfactory pathway, (in which case it would be difficult to understand the absence of clinical CNS disease, manifested so constantly in response to the intracerebral injection of the minutest amounts of virus) or whether the progression might still be limited to definite areas, the arrest however, occurring somewhat more posteriorly than in mice, but still in clinically "silent" zones.

This premise was tested in an experiment, recorded in Table IV, in which the rest of the brain was subdivided into several portions. After cutting away the anterior rhinencephalon, the cortex (no separation was then made between the neopallial and olfactory portions) was peeled away from the brain stem and portions of the parietal and occipital regions (including, of course, the piriform lobes and cornu Ammonis) were saved for tests. The diencephalon (including the pars optica hypothalami) and mesencephalon, the pons and medulla, and the cerebellum were the other regions examined for virus. 10 day old guinea pigs, which were given about 500,000 M.C.L.D. of the N. J. virus, were sacrificed at 18 hours, 3, 7, and 10 days after nasal instillation. The blood at all these intervals, and the brain tissue of those killed at 18 hours and 3 days showed no virus. In the guinea pig sacrificed on the 7th day abundant virus was demonstrated in the anterior rhinencephalon, the cortical regions, and the diencephalon and mesencephalon, while none was found in the pons and medulla and cerebellum. On

the 10th day a small amount of virus was still detectable in the anterior rhinencephalon, and diencephalon and mesencephalon, but none in any of the other regions.

These results seemed to indicate that the virus did not spread indiscriminately throughout the CNS, and another series of experiments were undertaken to localize more definitely the affected zones and the site of apparent arrest of progression.

Fate of Nasally Instilled Virus and Site of Arrest in CNS.—In the following experiment an attempt was made to ascertain (*a*) the fate

TABLE IV

Distribution of Nasally Instilled Vesicular Stomatitis Virus in the Central Nervous System of Young Guinea Pigs

Time after nasal instillation of virus 0.5× 10^{-4} M.C.L.D.	Presence of virus in						
	Blood	Anterior rhinencephalon	Parietal cortex	Occipital cortex	Diencephalon and mesencephalon (+ pars optica hypothalami)	Cerebellum	Pons and medulla
18 hrs.	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
3 days	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
7 days*	0, 0, 0	3, 5, 0	2, 3, 4	3, 3, 4	3, 3, 3	0, 0, 0	0, 0, 0
10 days†	0, 0, 0	6, 0, 0	0, 0, 0	0, 0, 0	5, 6, 0	0, 0, 0	0, 0, 0

* The guinea pig sacrificed on the 7th day had fever on the 6th (104.8°F.) and 7th (105.2°F.) days.

† The guinea pig sacrificed on the 10th day had fever on the 6th (104.6°F.) and seventh (104.5°F.) days.

Other legends as in preceding tables.

of the virus in the nasal mucosa especially in relation to its subsequent invasion of the CNS, and (*b*) whether or not its localization in the brain was in accord with a progression along the central connections of the olfactory neurons.

The age of the animal and the dose of virus were the controlled variables. The young guinea pigs were 7 to 10 days old and the old ones were at least 6 months of age. One group of each age received a nasal instillation of about 500,000 M.C.L.D. of the N. J. virus and another about 50,000 M.C.L.D. The nasal mucosa and various parts of the brain were tested for virus at 3 hours, 2, 5, and 7 days. The entire nasal mucosa was ground with alundum and 5 cc. of broth; after horizontal centrifugation of the suspension at about 2000 R.P.M. for 45 to 60 minutes,

the supernatant liquid and dilutions prepared from it, were injected intracerebrally in mice. The anterior rhinencephalon was obtained in the usual manner, but the remainder of the brain was dissected somewhat differently. Following along the very sharp and distinct rhinal fissure the cortex was easily separated into the neopallial portion (non-olfactory), and the archi- and paleo-pallial portions containing the piriform lobes, and cornu Ammonis (olfactory zones). The diencephalon (including the pars optica hypothalami) and mesencephalon were tested individually while the pons, medulla, and cerebellum were pooled.

The results shown in Table V may be summarized as follows: The *young guinea pigs* receiving *500,000* M.C.L.D. of virus exhibited: *3 hours later*, virus only in the undiluted suspension of nasal mucosa (*i.e.* about 100–200 M.C.L.D.) and none in any tested part of the brain; *2 days later*, only a trace of virus in the nasal mucosa but a considerable amount in the anterior rhinencephalon with none in any of the other regions of the brain; *5 days later*, small amount of virus in anterior rhinencephalon and none detectable in nasal mucosa or other regions of the brain; *7 days later*, an appreciable amount in the anterior rhinencephalon, and piriform and hippocampal regions with none found elsewhere in the brain or nasal mucosa.

The *young ones* instilled with *50,000* M.C.L.D. of virus exhibited: *3 hours later*, none in the nasal mucosa or brain; *2 days later*, abundant virus in the mucosa (present in the 10^{-2} but not in the 10^{-3} dilution) as well as in the anterior rhinencephalon; *5 days later* virus absent, or in smaller amount, in nasal mucosa, but present in considerable quantity in anterior rhinencephalon, piriform and hippocampal regions, and some in the diencephalon but not elsewhere in the brain; *7 days later*, none found in the nasal mucosa or any part of the brain with the exception of the diencephalon which contains a considerable amount.

The *old guinea pigs* given *500,000* M.C.L.D. of virus exhibited: *3 hours later*, none in nasal mucosa or brain; *2 days later*, abundant virus in nasal mucosa (10^{-2} dilution positive) and a trace in the anterior rhinencephalon, with none in the other regions of the brain; *5 and 7 days later*, a trace of virus in the nasal mucosa with none in any part of the brain.

The *old guinea pigs* given *50,000* M.C.L.D. of virus exhibited: *3 hours later*, none in nasal mucosa or brain; *2 days later*, none or undetermined small amount in nasal mucosa and none in brain; *5 days later*, trace in nasal mucosa and anterior rhinencephalon with none elsewhere in brain; *7 days later*, none in nasal mucosa or brain.

It appears, therefore, that as in mice (1), almost all the virus instilled into the nose disappears within a very short time, to such an extent that in three of the four guinea pigs tested within 3 hours none was detectable, indicating that of the 50,000 or 500,000 M.C.L.D. of instilled virus less than 100 M.C.L.D. remained fixed or in an infective state. It also seems clear that in both the young and the old guinea pigs there may be quite an appreciable increase in the amount of virus

in the nasal mucosa within the first 2 days which, however, disappears rapidly thereafter. When the results of the present experiment are

TABLE V

Fate of Nasally Instilled Vesicular Stomatitis Virus (N. J.) and Site of Arrest in Central Nervous System as Influenced by Amount of Virus and Age of Guinea Pigs

Age of guinea pigs	Amount of virus instilled	Time after nasal instillation	Guinea pig No.*	Presence of virus in									
				Nasal mucosa in 5 cc.				Anterior rhinencephalon	Piriform and hippocampus	Diencephalon + pars optica hypothalami	Mesencephalon	Pons, medulla, and cerebellum	Neopallium
				Undiluted	1:10	1:100	1:1000						
7–10 days	500,000 M.C.L.D.	3 hrs.	1	3, 7†	0, 0	n.t.	n.t.	0, 0	n.t.	0, 0	0, 0	0, 0	n.t.
		2 days	2	4, 0	0, 0	0, 0	0, 0	3, 3	0, 0	0, 0	0, 0	0, 0	"
		5 "	3	0, 0	0, 0	0, 0	0, 0	3, 0	0, 0	0, 0	0, 0	0, 0	0, 0
		7 "	4	0, 0	0, 0	0, 0	n.t.	3, 3	3, 4	0, 0	0, 0	0, 0	0, 0
	50,000 M.C.L.D.	3 hrs.	5	0, 0	0, 0	n.t.	n.t.	0, 0	n.t.	0, 0	0, 0	0, 0	n.t.
		2 days	6	2, 2	2, 2	2, 3	0, 0	2, 2	—‡	—	—	—	—
		5 "	7	—	0, 0	0, 0	0, 0	2, 3	2, 3	2, 0	0, 0	0, 0	0, 0
		7 "	8	0, 0	0, 0	0, 0	n.t.	0, 0	0, 0	2, 2	0, 0	0, 0	0, 0
Over 6 mos.	500,000 M.C.L.D.	3 hrs.	9	0, 0	0, 0	n.t.	n.t.	0, 0	n.t.	0, 0	0, 0	0, 0	n.t.
		2 days	10	2, 2	2, 2	2, 3	"	3, 0	"	0, 0	0, 0	0, 0	"
		5 "	11	5, 0	0, 0	0, 0	"	0, 0	"	0, 0	0, 0	0, 0	"
		7 "	12	(4), 0	0, 0	0, 0	"	0, 0	"	0, 0	0, 0	0, 0	"
	50,000 M.C.L.D.	3 hrs.	13	0, 0	0, 0	n.t.	"	0, 0	"	0, 0	0, 0	0, 0	"
		2 days	14	—	0, 0	0, 0	"	0, 0	"	0, 0	0, 0	0, 0	"
		5 "	15	0, 0	6, 6	0, 0	"	2, 0	"	0, 0	0, 0	0, 0	"
		7 "	16	0, 0	0, 0	0, 0	"	0, 0	"	0, 0	0, 0	0, 0	"

* None of the guinea pigs sacrificed for these tests exhibited any signs of disease.

† The brain of at least one sick or dead mouse in each group of two was cultured and subinoculated in animals in order to establish that the illness and death were due to virus.

‡ Dashes indicate that material was contaminated with pathogenic bacteria.

(4) = mouse died on 4th day but material was unsuitable for establishing cause of death.

combined with those of Table III, it is to be noted that among 16 old guinea pigs, a trace of virus was found only in the brains of two (lim-

ited to the anterior rhinencephalon), indicating that in spite of the multiplication of virus which may occur in the nasal mucosa it cannot as a rule invade the brain and that when it does get into the brain on occasions, it can apparently neither multiply nor progress beyond the anterior olfactory region. In the young guinea pigs, however, there is evidence of constant invasion of the brain, the virus becoming detectable in the anterior rhinencephalon on the 2nd day at which time the other regions of the brain show none. It then spreads posteriorly apparently in accord with a definite order localizing in the piriform and hippocampal regions but not in the neopallial regions of the cortex, and occasionally in the diencephalon (or only the pars optica hypothalami) but apparently not beyond. The finding of virus only in the diencephalon in the brain of one guinea pig on the 7th day merely suggests the possibility that it may disappear last from the areas which are last to be involved. This type of localization, however, is completely in accord with progression of the virus within the neurons of the olfactory pathway and not at all in agreement with a spread of the virus in an open system. It can also be inferred, therefore, that the absence of apparent CNS disease in nasally instilled guinea pigs may be effected by the same mechanism which determined the resistance of old mice. The difference between the two species is in (*a*) the sites at which the progression of nasally instilled virus is arrested, and (*b*) in the fact that 7 to 10 day old guinea pigs appear to possess "barriers" which are acquired by mice only at a much later age.

Spread of Virus after Intramuscular or Pad Inoculation.—Although in past investigations by others, large numbers of guinea pigs have been injected into the pads with the vesicular contents from horses and cattle, or with the pad passage or more recent brain passage strains, there is no record of any paralysis or other signs of CNS disease occurring in these animals. When the present investigation was begun, twelve guinea pigs, approximately 1 month old, were injected intracutaneously and subcutaneously in the pad of one posterior extremity with mouse brain virus and one developed typical flaccid paralysis of the inoculated posterior extremity on the 5th day followed by paralysis of the opposite leg, and ascending paralysis resulting in death on the 9th day after inoculation. It is regrettable

that no search for virus was made, although no other cause for these signs was found. In view of this unexpected finding many other guinea pigs, and particularly very young ones, were inoculated in the same manner without our ever again observing any evidence of CNS disease.

TABLE VI

Spread of Vesicular Stomatitis Virus (N. J.) after Intramuscular or Pad Inoculation

Site of Inoculation	Age and average weight of guinea pigs	Time after inoculation	Presence of virus in: Blood	Spleen	Regional lymph nodes	Right sciatic nerve	Right sacral and lumbar ganglia	Spinal cord	Left sacral and lumbar ganglia	Left sciatic nerve
		days								
Intracutaneously and subcutaneously in right pad (about 10^6 M.C.L.D.)	About 1 mo.; 300 gm.	3	0, 0, 0	n.t.	n.t.	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
		6	0, 0, 0	"	"	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
		10	0, 0, 0	"	"	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
	About 1 mo.; 300 gm.	6	0, 0, 0	"	"	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
		10	0, 0, 0	"	"	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
Intramuscularly in right calf muscles (about 10^5 M.C.L.D.)	10 days; 167 gm.	2	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
		4	0, 0, 0	0, 0, 0	2, 3, 3	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
		8	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
	Over 3 mos.; 580 gm.	2	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
		4	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
		8	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0

The following tests were undertaken to determine whether or not the virus injected intramuscularly or into the pads, was capable of invading the CNS along the nerves supplying the inoculated sites.

One group of 1 month old guinea pigs was given about 10^6 M.C.L.D. of mouse brain virus (N. J.) intracutaneously and subcutaneously in the right pad. Two animals were sacrificed at 3, 6, and 10 day intervals and the structures, indicated

in Table VI, were pooled and injected intracerebrally in mice. Three other groups of guinea pigs (young and adult) were given approximately the same amount of virus intramuscularly in the right posterior extremity, and various tissues were tested at 2, 4, 6, 8, and 10 day intervals.

The results shown in Table VI indicated that in none of these animals was virus found in the blood, peripheral nerves, spinal ganglia, or spinal cord. Virus was detected but once and that was in the regional lymph nodes of a 10 day old guinea pig, 4 days after intramuscular injection. It was clear, therefore, that in young and old guinea pigs, as in old mice, absence of manifest CNS disease following this form of peripheral inoculation was associated with an inability of the virus to invade the nervous system. In old mice it will be recalled, this was associated with inability of the virus to multiply at the site of inoculation. Although vesicular stomatitis virus is known to produce definite vesicular lesions in the pads of guinea pigs, and, therefore, presumably to be capable of local multiplication, it was nevertheless desirable to establish this fact beyond question particularly for the virus used in these experiments, which has undergone over 100 brain to brain passages in mice.

Twelve 8 to 10 day old guinea pigs and an equal number of old animals weighing on the average 1000 gm. each were given about 10^6 M.C.L.D. of the N. J. virus into the pad of one posterior extremity, part by "tunneling" and the remainder subcutaneously, and a similar volume of plain broth in the other for control. Only the pads inoculated with virus developed lesions; these were distinct on the 3rd day and were generally less marked in the young than in the old. Histological sections of some of the pads revealed the characteristic picture including intranuclear inclusions and necrosis of epithelial cells. Animals of each group were sacrificed at 2, 22, and 72 hours after inoculation. The pads inoculated with virus were washed with alcohol and ether, dissected away, and ground up with alundum and broth. The pad of an old guinea pig, weighing usually 0.3 gm., was ground up with 6 cc. of broth, that of a young one, weighing 0.1 gm., with 5 cc. of broth. The suspensions were centrifuged at about 2000 R.P.M. for 45 to 60 minutes, and the clear supernatant liquids (designated in the old as the 1:20 dilution and for the young as the 1:50) and further dilutions of them were injected intracerebrally in mice.

The results of this experiment (Table VII) leave no doubt as to the local multiplication of the virus. It is clear, therefore, that local multiplication does not in itself determine the capacity of this virus to invade the peripheral nerves.

Effect of Intrasciatic Injection of Virus.—Inoculation of the virus directly into the sciatic nerve of resistant mice resulted in a fatal ascending myelitis in six of eleven animals, indicating that virus progression along peripheral nerve fibers was possible and that the chief barrier to invasion of the CNS was in some structure or structures at the site of intramuscular or pad inoculation (1).

To determine whether or not the same was true for guinea pigs, twelve of them were given a 10 per cent suspension of N. J. virus into the right sciatic nerves. An equal number of guinea pigs which, for control, were injected intramuscularly, in the neighborhood of the same region of the sciatic nerve, remained well.

TABLE VII

Local Multiplication of Vesicular Stomatitis Virus (N. J.) in Pads of Young and Old Guinea Pigs

Age and average weight of guinea pigs	Time after inoculation of about 10^4 M.C.L.D.	Dilution of pad suspension						
		1:20	1:50	1:100	1:1000	1:10,000	1:100,000	1:1,000,000
	hrs.							
8–10 days; 121 gm.	2	n.t.	3, 5	n.t.	0, 0	0, 0	n.t.	n.t.
	22	"	2, 2	"	3, 5	8, 9	"	"
	72	"	4, 5	"	5, 10	0, 0	0, 0	0, 0
Over 3 mos.; 1000 gm.	2	0, 0	n.t.	0, 0	0, 0	0, 0	n.t.	n.t.
	22	2, 3	"	2, 3	3, 4	2, 5	"	"
	72	4, 4	"	0, 0	5, 7	0, 0	0, 0	0, 0

Four of the twelve guinea pigs (Table VIII) receiving the virus in the sciatic nerve, developed typical flaccid paralysis of the posterior extremities, which ascended and caused death in three instances, the fourth animal having been sacrificed for virus tests. Some of the guinea pigs without nervous signs exhibited fever (104–106°F.) but no spread of virus was demonstrable in association with it. Tests for virus in a guinea pig dying with paralysis revealed its presence in the lumbar and cervical portions of the cord, the medulla, and brain, but not in either one of the sciatic nerves. The failure to detect virus in the inoculated sciatic nerve at the time of paralysis and its presence in the lumbar cord, observed also in another guinea pig, duplicates the experience with this virus in old mice and its probable significance has already been discussed (1).

It is clear from these experiments that vesicular stomatitis virus injected directly into a peripheral nerve like the sciatic can invade

TABLE VIII

Effect of Intrasciatic Injection of Virus

Age and average weight	Guinea pig No.	Result	Presence of virus in: Right sciatic nerve	Left sciatic nerve	Lumbar cord	Cervical cord	Medulla	Brain	Spleen
10–12 days; 200 gm.	1	Fever 5th and 6th days; paralysis of posterior extremities 5th day; dead 7th day	0, 0	0, 0	2, 2	3, 3	3, 3	2, 2	n.t.
	2	Fever* 6th, 7th, 8th, 10th days; remained well							
10 weeks; 500 gm.	3	Fever 5th, 7th, 10th days; paralysis of posterior extremities 5th day; encephalitic signs, 13th day; dead 15th day							
	4	Fever 10th, 11th, 12th days; remained well							
About 6 mos.; 600 gm.	5	Fever 7th, 10th, 11th, 12th days; remained well							
	6	Fever 11th, 12th, 13th days (105–106°F.); no nervous signs; sacrificed 13th day	0, 0	0, 0	0, 0	0, 0	0, 0	0, 0	0, 0
6–10 wks.; 350–500 gm.	7	No fever; remained well							
	8	" " " "							
	9	Fever 3rd, 4th, 6th days; paralysis right posterior extremity 5th day; paralysis both posterior extremities 6th day; dead 7th day							
	10	No fever; paralysis of both posterior extremities 6th day; no change 7th day; sacrificed 7th day	0, 0, 0	0, 0, 0	3, 5, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
	11	No fever; remained well							
	12	Remained well							

* Fever refers to a distinct rise in temperature of 1.5°F. or more over the preceding level and reaching at least 104°F.

the CNS of guinea pigs which it is incapable of accomplishing after inoculation into a site supplied by this nerve. In view of the fact also that the CNS is highly susceptible and that the virus can multiply in the area of peripheral injection affecting tissue cells supplied by these peripheral nerves, one is forced to consider, by a process of elimination, that the terminal, specialized nerve endings may constitute the real barrier to invasion of the CNS.

DISCUSSION

A number of observations made in the present investigation indicate that a neurotropic virus to which the CNS of two species is equally susceptible may, after peripheral inoculation, cause encephalitis or myelitis in one but not in the other, the difference being determined by variations in the character of the nervous pathways along which the virus must spread. When vesicular stomatitis virus is introduced directly into the brain, the minimal dose (1 M.C.L.D.) can produce encephalitis in young guinea pigs as readily as in young mice. On the other hand, while 100 to 1000 M.C.L.D. instilled intranasally in young mice, invariably gives rise to a fatal encephalitis, 1000 times that amount is, with very rare exceptions, entirely unassociated with any clinical signs of CNS involvement. Since the nasally instilled virus, nevertheless, regularly invades the CNS of young guinea pigs and spreads through the olfactory regions of the brain along pathways which it has been shown to utilize in susceptible mice, it would appear that some variation in these nervous pathways is responsible for the arrest of its progression in the terminal olfactory areas or diencephalon. After inoculation into tissues supplied by spinal nerves (*e.g.* sciatic) the virus undergoes local multiplication in young guinea pigs as in young mice, but causes myelitis only in the latter while in the former it fails to invade the nervous tissue altogether. The fact that direct intrasciatic injection is frequently followed by a fatal ascending myelitis tends to eliminate the peripheral nerves themselves as the barriers to invasion of the CNS and forces consideration of a variation in structures, such as the myoneural junctions or other specialized nerve endings, through which is effected the intimate relationship between the axis cylinders and the inoculated tissues. The rôle of localized barriers in hosts of different age or species in preventing or

TABLE IX

Localized Barriers as a Factor in Preventing or Arresting Infection of the Central Nervous System (Vesicular Stomatitis Virus)

Host	Route of inoculation	Susceptibility of young and old	Probable site of barrier
Mouse	Intracerebral	Both equally susceptible	0
	Intramuscular Subcutaneous, pad	Young, regularly myeloencephalitis Old, 100 per cent resistant	Muscle or myoneural junction; epithelium or specialized nerve endings
	Intraocular	Young, regularly encephalitis Old, more than 90 per cent resistant	Retina
	Intranasal	Young, regularly encephalitis Old, resistance varies from 50 to 90 per cent	In CNS, anterior olfactory region (between 2nd and 3rd olfactory neurons?)
Guinea pig	Intracerebral	Young guinea pigs and mice equally susceptible, old guinea pigs somewhat less	0
	Intramuscular Subcutaneous Intracutaneous	Young and old resistant	Myoneural junction and specialized nerve endings
	Intranasal	Young and old resistant	Young, (*a*) between olfactory cortex and remainder of brain (*b*) between diencephalon (or pars optica hypothalami) and remainder of brain Old, generally between nasal mucosa and CNS

arresting infection of the CNS with vesicular stomatitis virus is summarized in Table IX.

There are many other instances in nature where a virus is highly neuroinvasive in one species and apparently not at all (or very rarely) in another. As a classical example one may cite the virus which, while causing only herpes simplex in human beings, will, when transferred to the skin, cornea, or mucous membrane of the rabbit, cause not only local lesions like those in man, but also clinically apparent and fatal disease of the CNS. Pseudorabies (4) and B virus (5, 6) which are pantropic and highly neuroinvasive in the rabbit, will cause encephalitis when injected intracerebrally but not peripherally (skin, muscle) in the *rhesus* monkey; this is associated with a loss of the capacity to produce lesions in non-nervous tissue of the monkey in the case of pseudorabies but not of B virus. That variation in the character of the specialized nerve endings in different species may be involved here, as in the case of vesicular stomatitis virus, receives some support in Hurst's finding that intrasciatic injection of pseudorabies virus in the *rhesus* monkey frequently causes myeloencephalitis (4).

The observations made in this series of studies on vesicular stomatitis virus in different hosts may supply a pattern for at least one type of inapparent infection of the CNS. It is generally assumed, for example, that the majority of the human population are subject to inapparent infection with the virus of poliomyelitis, while only rare individuals exhibit the clinically apparent form of the disease. Faber (7) has thus postulated on theoretical grounds that poliomyelitis virus in man, spreading axonally along the olfactory pathways, might be halted in its progression in silent zones of the CNS in the majority of instances. That such a thing is possible and actually occurs with another neurotropic virus is evident from the demonstrated behavior of vesicular stomatitis in guinea pigs which seems to offer a remarkable parallel for many of the manifestations of poliomyelitis in man. There are the rare, individual guinea pigs which after nasal instillation exhibit clinical signs of CNS disease, while in the majority which appear well there is, nevertheless, transitory multiplication of the virus in the nasal mucosa with involvement of definitely limited zones in the brain; and to accentuate the parallel

even further it may be stated here that antiviral bodies regularly appear in the blood of all guinea pigs regardless of the extent of CNS involvement. (Detailed studies on the immune response to arrested infection with vesicular stomatitis virus will be presented in a future communication.) Other patterns of inapparent infection of the CNS, which have been described recently, must also be considered. Burnet (8), for example, showed that louping ill virus which regularly causes encephalitis in the mouse, is followed by no signs of disease when the virus is injected by intracerebral or peripheral routes in young or old rats. After nasal instillation in rats there appears, nevertheless, to be local multiplication of the virus and an invasion of the brain that is generally limited to the olfactory bulbs. The observations of Webster and Clow (9) on mice with a high inborn resistance to the virus of St. Louis encephalitis demonstrate still another type of clinically inapparent infection of the CNS; intracerebral and peripheral inoculation are equally harmless in these mice, and nasal instillation is followed by as widespread an invasion of the brain as in the susceptible animals. The absence of nervous signs here is correlated with a distinctly lower level of virus multiplication throughout the CNS, rather than with localized barriers to virus progression.

SUMMARY AND CONCLUSIONS

Peripheral inoculation of vesicular stomatitis virus is constantly followed by myelitis or encephalitis in young mice, but not in young (or old) guinea pigs. The cause of this variation was elucidated by investigating the fate of the virus after inoculation by a number of different routes.

Direct intracerebral injection of minimally infective amounts of virus was found to be equally fatal for young mice and young guinea pigs, indicating that the central nervous system as a whole was as easily injured by the virus in one species as in the other.

The events following nasal instillation of the virus varied in young and old guinea pigs. While there appeared to be a transitory multiplication of virus in the nasal mucosa of both young and old, the central nervous system was regularly invaded only in the young. In these, virus was first found only in the anterior rhinencephalon; later it spread to the piriform and hippocampal (olfactory regions)

but not to the neopallial portions of the cortex, and the only other area to exhibit virus was the diencephalon (including the pars optica hypothalami), where its further progression was apparently arrested.

Absence of central nervous system disease following inoculation into sites supplied by spinal nerves (*e.g.* sciatic) was found to be due to inability of the virus to invade the nerves.

Since direct intrasciatic inoculation frequently led to a fatal ascending myelitis, it was evident that the central nervous system could be invaded along the spinal nerves, and that they did not constitute the main barrier. Furthermore, since multiplication of virus was demonstrated in tissues supplied by the spinal nerves, a process of elimination made it seem possible that the specialized, terminal nerve endings might be the structures which prevent the progression of the virus from the infected tissues to the axons and hence also to the central nervous system.

7 day old guinea pigs (or guinea pigs as a species) were thus found to possess much the same type of barriers to the progression of peripherally inoculated vesicular stomatitis virus as are acquired by mice at a considerably later age.

In a discussion of the present data, they have been correlated with known variations in neuroinvasiveness of other viruses and their bearing on the nature of inapparent or subclinical infections of the central nervous system has been considered.

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SERUM SODIUM, POTASSIUM AND CHLORIDE AFTER SUPRARENALECTOMY IN CATS WITH DIABETES INSIPIDUS

By CHARLES A. WINTER, Ph.D., E. G. GROSS, M.D., and W. R. INGRAM, Ph.D.

(From the Departments of Physiology, Pharmacology and Anatomy, State University of Iowa, Iowa City)

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Many investigators (1–11) have shown that changes occur in the concentration of serum electrolytes during suprarenal insufficiency. There is evidence that these changes are due to shifts of such ions within the body fluids, and some workers (2, 3) have suggested that the kidney may be one of the sites of action of the suprarenal cortical hormone regulating serum electrolytes. Animals with diabetes insipidus show a relatively unrestrained loss of fluids *via* the kidney due to lack of the antidiuretic hormone of the posterior lobe of the pituitary. It has been maintained by certain clinical investigators that patients with this disease also show disturbances in the metabolism and excretion of sodium and chloride, and it is well known that administration of posterior pituitary substance promotes the excretion of salt. It is, therefore, of interest to observe the changes in serum sodium, potassium and chloride following removal of the suprarenals in cats with diabetes insipidus.

Methods

Adult cats were used, without regard to sex. The general feeding and care of the animals have been described elsewhere (12). Diabetes insipidus was produced by interruption of the supraoptico-hypophyseal tract, using the Horsley-Clarke stereotaxic instrument; the lesions so produced involve a portion of the hypothalamus and result in atrophy of the pars nervosa of the pituitary (12). Suprarenals were removed in two stages, using a dorsal approach. It was early found that the survival time following suprarenalectomy was considerably shorter in the cats with diabetes insipidus than in control animals; therefore, some of the animals were maintained for a short time following the second suprarenal operation

with active extracts of the suprarenal cortex[1] to insure complete recovery from the effects of the operation itself.

Blood for analysis was obtained from the unanesthetized animal by puncture of the left ventricle. Chlorides were determined by the method of Wilson and Ball (16), potassium by Shohl and Bennett's procedure (17) and sodium by the Butler and Tuthill method (18). The control blood specimens (called insufficiency none in Table I) were usually obtained just before the removal of the second suprarenal; those called insufficiency pronounced in Table I were obtained within a few hours of death, when the animal was obviously *in extremis*.

RESULTS

The results are summarized in Table I. There are two groups of controls: one in which the animals were left intact except for suprarenalectomy (called normal controls in Table I), and the other group (two animals) which received a hypothalamic lesion, but did not develop the polyuria and polydipsia of diabetes insipidus. Histological sections show the supraoptico-hypophyseal tracts and posterior lobes of the latter two cats to be normal, while in those cats which developed polyuria, the tracts are interrupted and the posterior lobes atrophic. The daily urine output of our normal cats is about 100 to 125 cc.; the polyuric cats excrete from 300 to 600 cc. a day on the average, except DI-23, which averaged only about 200 cc. The urine volume in both the control and the polyuric cats was somewhat reduced following the removal of the second suprarenal.

Although the data on chloride are more complete than on sodium or potassium, Table I clearly shows that the only change in serum electrolytes consistently shown in all groups of animals following suprarenal removal is an increase in the concentration of serum potassium. The average serum potassium value found for all cats before bilateral suprarenalectomy was 24.8 mg. per cent. Control cats after the development of symptoms of suprarenal insufficiency showed an average serum potassium figure of 36.0 while the polyuric cats in suprarenal insufficiency have a mean serum potassium of 34.0.

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[1] The suprarenal cortex extract was obtained through the courtesy of Dr. David Klein, of the Wilson Laboratories, Chicago, Illinois, and Dr. Oliver Kamm of Parke, Davis and Company, Detroit, Michigan. The authors are also grateful to Dr. W. I. Evans of the Department of Anatomy for technical assistance in some of these experiments.

TABLE I

Cat No.	Date	Gross symptoms of suprarenal insufficiency	Serum electrolytes			Remarks
			Potassium	Sodium	Chloride	
			mg. per cent	mg. per cent	mg. per cent	
NA	June 24	None	22.9	—	—	Normal control
NA-2	" 21	"	27.9	350	424	" "
NA-1	" 8	"	—	—	419	" "
	" 21	Moderate	34.7	280	346	
	" 26	Slight (on inadequate extract)	26.2	297	352	
	" 30	Pronounced	30.5	273	344	
NA-3	Sept. 8	None	26.4	363	417	Normal control
	" 16	Pronounced	34.4	303	390	
DI-29	Aug. 16	None	24.7	338	441	Control cat with hypothalamic lesion, but no polyuria
	" 27	Pronounced	39.3	240	381	
DI-31	" 17	None	22.9	349	415	" "
	" 21	Moderate	26.1	313	387	
	" 23	Pronounced	41.0	300	368	
DI-11	May 6	None	—	—	404	Marked polyuria
DI-7	" 6	"	—	—	409	" "
	" 9	Pronounced	—	—	442	
DI-8	" 28	None	—	—	406	Marked polyuria
	" 31	Slight	—	—	403	
	June 1	Pronounced	—	—	409	
DI-14	" 3	None	—	—	414	Marked polyuria; cortin June 3 to 11 in gradually reduced doses
	" 9	"	—	—	415	
	" 13	Pronounced	34.4	328	423	
DI-26	Aug. 11	None	22.9	340	427	Marked polyuria; cortin Aug. 11 to 15 in gradually reduced doses
	" 18	Pronounced	34.4	337	397	
DI-32	Sept. 8	None	27.6	334	419	Marked polyuria; cortin Sept. 8 to 12
	" 15	Pronounced	38.5	316	417	
DI-23	Aug. 10	None	23.4	325	423	Slight polyuria; cortin Aug. 8 to 16
	" 20	Moderate	28.7	310	400	

Both groups of control cats present the typical picture of suprarenal insufficiency, the increased serum potassium being accompanied by a decrease in serum sodium and chloride, but in the polyuric animals these changes are slight or absent. The average serum sodium value for all animals before suprarenalectomy is 343 mg. per cent; during suprarenal insufficiency, the average figures are 279 mg. per cent for the control cats and 323 mg. per cent for the polyuric animals. For the three control cats for which the sodium figures both before and after suprarenalectomy are available, the change in serum sodium level ranges from −49 to −98 mg. per cent, while in the polyuric animals the range is from −3 to −18 mg. per cent. The average serum chloride value before suprarenalectomy is 418 mg. per cent, and during suprarenal insufficiency the average for the control animals is 366 mg. per cent and for the polyuric animals 415 mg. per cent. The change in chloride levels during suprarenal insufficiency as compared to the concentration before suprarenal removal ranges from −27 to −67 mg. per cent for the control animals, and from −30 to +33 for the polyuric cats. From these figures, it is evident that the behavior of serum electrolytes following bilateral suprarenalectomy is markedly different in cats with diabetes insipidus than in the control animals.

DISCUSSION

Since the work of Loeb (1, 2) and Harrop (3), the view has been widely held that the vital hormone of the suprarenal cortex is primarily a regulatory mechanism for sodium. The beneficial effect of sodium therapy in suprarenal insufficiency (1–6) supports this view. Much recent work, however, indicates that such an explanation for suprarenal insufficiency may be inadequate. Truszkowski and Zwemer (7) suggest that the primary defect may be in potassium metabolism. Swingle and coworkers have shown (8, 9) that in dogs on a sodium- and chloride-free diet, withdrawal of cortical extract leads to symptoms of suprarenal insufficiency without significant change in serum sodium or chloride. Swingle (9) considered the changes in serum potassium in these animals to be insignificant, but his data show an increase of about the same order of magnitude which we find. In suprarenalectomized-nephrectomized rats, Ingle, Nilson

and Kendall (10) found the concentrations of sodium and chloride in the serum normal, but the potassium increased. Nilson (11) reported that acute symptoms of suprarenal insufficiency may be produced in suprarenalectomized dogs either by a low intake of sodium and chloride or by a high intake of potassium; in such animals, changes in blood urea, sugar, sodium, chloride and hematocrit value may be found, but only an increased potassium content was found to be characteristic. In the opossum and marmot, suprarenal insufficiency is not accompanied by lowered values for sodium or chloride, but rather by an increase, according to Silvette and Britton (13, 14); these authors have not reported on potassium changes.

The survival time following suprarenalectomy is shorter for the polyuric than for the non-polyuric cats. It might therefore be argued that the usual decrease in serum sodium and chloride does not appear in the animals with diabetes insipidus simply because there has not been time enough for such a change. To meet such possible objection, we have prolonged the survival time of several of the polyuric animals to a week to 10 days with extract, and in two of the animals (DI-14 and DI-26) the extract dosage was gradually tapered off so that the suprarenal insufficiency would develop gradually, as it does in non-polyuric animals. Furthermore, cat DI-23 survived 4 days after complete withdrawal of extract, and cat DI-8 survived the operation 4 days although no extract was administered. In all cases, the external symptoms were the same in the polyuric suprarenalectomized animals as in those with uncomplicated suprarenal insufficiency; there was lack of appetite, loss of skeletal muscle tone, ataxia (especially in the hind limbs), gradual loss of interest in the surroundings, and in the terminal stages occasional mild convulsions. At autopsy, hyperemic or hemorrhagic areas were often found in the stomach and intestinal wall. The only difference noted between the two groups of animals was the rapidity with which the symptoms developed; the onset of suprarenal insufficiency was unquestionably hastened by the presence of diabetes insipidus.

Our data indicate that an increase in concentration of potassium in the serum is a more consistent characteristic of suprarenal insufficiency than a decrease of sodium, and that in the absence of normal functioning of the posterior lobe of the pituitary the usual

decrease in serum sodium and chloride does not occur. They do not, however, show that a disturbance in potassium metabolism or distribution is the sole or the primary disturbance in suprarenal insufficiency. MacKay, Bergman and MacKay (15) have recently shown that nephrectomized rats survive much longer than nephrectomized-suprarenalectomized rats, although the potassium content of the serum reaches a much higher level in the former than it attains in the terminal stages of the latter.

Our results suggest the possibility of some sort of interrelationship between the suprarenal cortex and the posterior lobe of the pituitary, so far as salt metabolism or distribution is concerned. Karlson and Norberg (19) and Debré, Marie, Nachmansohn and Bernard (20) have reported tests performed on patients with diabetes insipidus which indicate that in this condition there is diminished ability to concentrate sodium chloride in the urine. When unusual amounts of sodium chloride were added to the diet, the ability to concentrate the salt was improved after administration of pituitrin. The results of Smith and MacKay (21) are somewhat at variance with these. The latter report that while pituitrin increased the sodium and chloride output of normal persons so that the balance became negative, equivalent doses given to a subject with diabetes insipidus caused no increase in sodium chloride excretion nor did the sodium balance become negative; salt feeding was not attempted in these experiments, however. The observations of the two groups of workers first mentioned might lead to the suggestion that serum sodium and chloride remained normal in our animals because the kidneys of the cats with diabetes insipidus failed to respond to lack of suprarenal cortical hormone by increased clearance of these substances. Silvette (22) has found that pituitrin facilitates salt excretion in suprarenalectomized opossums receiving extra salt in the diet. Our animals, however, did not receive unusual amounts of salt, and appear, under ordinary circumstances, to run quite constant chloride balances. It appears that the suprarenal cortex and the posterior lobe interact in such a way that serum sodium and chloride remain unaffected in the absence of both principles. However, as we have only two control animals with hypothalamic lesions without diabetes insipidus, the possibility is not excluded that the effect which we observe may be due to the

hypothalamic lesion itself rather than to the diabetes insipidus. Further work along these lines is indicated. Nevertheless, it is clear that the level of sodium and chloride in the serum is not necessarily a measure of suprarenal insufficiency.

SUMMARY

The external symptoms of suprarenal insufficiency in cats with diabetes insipidus are the same as in those animals with only the suprarenals removed, except that the symptoms develop more rapidly in the former. The serum electrolyte changes, however, are different; there is no consistent or marked decrease in the concentration of sodium or chloride following suprarenalectomy in cats with diabetes insipidus, but there is the usual increase in the concentration of potassium. It is suggested that this indicates that changes in sodium are less characteristic of suprarenal insufficiency than are disturbances of potassium metabolism or distribution. A possible interrelationship between the suprarenal cortex and the posterior lobe of the pituitary as salt-regulating mechanisms is discussed.

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IRON METABOLISM IN EXPERIMENTAL ANEMIA

"Availability of Iron"

By P. F. HAHN, Ph.D., and G. H. WHIPPLE, M.D.

(From the Department of Pathology, The University of Rochester School of Medicine and Dentistry, Rochester, N. Y.)

(Received for publication, November 4, 1937)

"Availability of iron" is a term much used in current writings, particularly in those articles dealing with various forms of anemia.

In 1930 Hill (9) introduced the dipyridyl method for the determination of iron. When dipyridyl is added to a buffered suspension of animal tissue or foodstuff which has first been reduced with hydrosulfite, a red color is produced which is due to the formation of an iron complex. Iron combined as hematin is said not to give this reaction.

Elvehjem and Hart and their associates (7, 17) claim that the dipyridyl reagent reacts only with the iron which is not bound in complex form (non-hematin iron), and that since hematin iron is not available for hemoglobin production, the amount of iron in the food which can be utilized by the body is measured by this method. We propose to offer evidence that such a premise is untenable.

Methods

The dipyridyl method for determination of "available iron" was as described by Shackleton and McCance (16) with the exception that in addition to filtration of the extracted tissue suspension, centrifugalization was used to produce a clearer solution for final comparison. The method as applied to tissues and foodstuffs is open to criticism. Many of the failings have been noted by Shackleton but it would seem that little has been done to correct them. Many unjustified assumptions must be made and certain weaknesses in the procedure shake one's confidence in the method accuracy. Inaccuracies may be introduced due to interference of natural pigments, use of a comparator in the final estimation, and superior buffering of some natural materials over the acetate-acetic acid. We include below a few values obtained by the method as used in different bands (16, 17, 18) to illustrate the validity of these criticisms (Table 1).

The method for determination of total iron was a modification of that originally reported elsewhere (6). The material was ashed by the wet procedure involving sulfuric and perchloric acids. After diluting to a known volume aliquots were transferred to 100 ml. centrifuge tubes and neutralized with 40 per cent NaOH using phenolphthalein as an indicator. The standards at this point were treated with concentrated sulfuric acid and all samples and standards received the same amount of NaOH, acid being added as found necessary in each case. In this way

TABLE I

	Material	Total Fe	"Available Fe"
		mg. per cent	*per cent*
Elvehjem, Hart, and Sherman..........	Pig's liver	—	60
Shackleton and McCance.............	" "	20	80
Hahn............................	" "	21	48
Elvehjem, Hart, and Sherman..........	Beef muscle	—	50
Shackleton and McCance.............	" "		10–25
Shackleton and McCance.............	Apricots (dry)	4.08	98
" " "	" (fresh)	0.37	95
Hahn............................	" (dry)	4.2	114
Smith and Otis.....................	" (dry)	—	58
Sturgis and Farrar..................	Salmon bread	—	50
Hahn............................	Complete diet*	2.4	58
Shackleton and McCance............	Salmon	0.89	94
Hahn............................	"	0.40	120
Elvehjem, Hart, and Sherman..........	Spinach	—	25
Shackleton and McCance.............	" (raw)	2.96	68
" " "	" (cooked)	4.15	57

* Salmon bread 200; Klim 20; salmon 75; salt mixture 1 gm.

the contamination of the standards and samples with iron by reagents was to a great extent constant in amount. After standing overnight they were centrifugalized for 20 minutes at about 2500 R.P.M., the supernatant liquid was poured off and the precipitate was dissolved in 2 ml. of 2/3 N HCl. The solution was transferred to a 25 ml. volumetric flask and the tube rinsed with 7 ml. of M/5 potassium acid phthalate solution and the rinsings added to the flask. Further rinsing of the tube was done with about 8 ml. of distilled water. 2 ml. of 0.2 per cent solution of 7-iodo-8-hydroxyquinoline-5-sulfonic acid were added and

after making to volume comparison was made in a colorimeter. If the readings of the unknowns varied by more than 10 per cent from the standards a correspondingly larger or smaller aliquot was taken and the determination repeated. This has been shown to be necessary due to the difference in color obtained at various iron concentrations as has been mentioned elsewhere (6).

Care of dogs, standard diets, and method procedures related to the anemic dogs have been described in detail (21).

EXPERIMENTAL OBSERVATIONS

A number of determinations were carried out with individual food materials as well as with a complete salmon bread diet. Comparison of the resulting values for "available iron" with those reported by others bears out the criticisms listed above. The procedure apparently does not give similar results in different hands.

TABLE 2

Dog No.	Tissue	Total Fe	"Available Fe"	"Available Fe"
		mg. per cent	*mg. per cent*	*per cent*
34-5	Liver	1.24	0.5	40
	Heart	2.91*	1.0	34
	Spleen	7.18†	1.6	22
30-115	Liver	3.16	1.4	44
	Heart	2.74*	1.4	51

* About 1/3 to 1/2 of this represents muscle hemoglobin iron (6).

† Some of the iron in this tissue is explained by blood not removable by perfusion.

Dogs with long continued anemia due to blood withdrawal fed a diet poor in iron will show complete exhaustion of all iron reserve stores (6). The iron remaining in the liver and other tissues appears to be essential to cell life, cannot be further reduced by any of the procedures tried, and cannot be drawn upon to produce new hemoglobin. This tissue or "parenchyma iron" may be in part cytochrome iron and it is probably not in inorganic form.

Table 2 gives the analysis of various organs of two dogs whose iron reserve had been completely exhausted by long continued anemia due to blood loss plus a diet very low in iron. The figures are self-

explanatory. Under ether anesthesia the viscera and other tissues were rendered blood-free by viviperfusion.

DISCUSSION

Iron salts frequently used (ferric and ferrous chloride), and presumably any ionizable iron salts, react quantitatively with the dipyridyl reagent and therefore are spoken of as 100 per cent available. Yet when fed to our standard anemic dogs in optimum amounts these salts are but 30 to 40 per cent available—using the same terms, and similar amounts of iron as given in meat or liver. Numerous experiments in dogs (20) given 40 mg. of iron *by mouth* per day for 14 days (total 560 mg. of iron) show a *net production* of 50 to 55 gm. new hemoglobin above the basal ration control output. If given *by vein* this same dose of iron will yield a 100 per cent return of hemoglobin, or 160 to 170 gm. hemoglobin net output. Therefore, this iron is 100 per cent "available" by the dipyridyl method and by intravenous test, but only about 35 per cent "available" when given by mouth. Furthermore, when larger doses of iron are given by mouth the "availability" falls off rapidly and with 400 mg. iron per day, or ten times the optimum dose, we record only double the production of new hemoglobin (95 to 100 gm. net output). The term "available iron" boils down to iron not in the form of hematin compounds, and what will happen to this "available iron" in the intestinal tract has no relation to the dipyridyl test but is conditioned by a great variety of factors, some known (4, 8) and others not recognized as yet.

From work in this laboratory it has been found in a long series of experiments that the feeding of 300 gm. of pig liver per day to a standardized anemic dog over a 2 week period will result in the production of an extra 95 gm. of hemoglobin over and above the control level. Assuming an average liver iron content of 20 mg. per cent, the extra iron intake would amount to 840 mg. during the feeding period. The iron corresponding to the surplus hemoglobin (95 gm.) is 318 mg. Therefore there is a return of 38 per cent of the metal as fed. This is somewhat less than the figure for the availability of iron in pig liver recorded above (48 per cent) as determined by one of us using the dipyridyl method, and considerably less than the values recorded by others, 60 per cent (17) and 80 per cent (16). The

physiological availability of iron again does not correspond to the "availability" as determined by dipyridyl.

Apricot feeding presents experimental data of interest in this connection. Feeding 100 gm. of dried apricots a day for 2 weeks to the standard anemic dog results in a surplus hemoglobin production of 42 gm. The iron contained is 100 per cent "available" (16) but amounts to only 4 mg. per 100 gm. of apricots—or 56 mg. per 14 days. If the iron was quantitatively changed to hemoglobin it would account for only 19 of the 42 gm. of hemoglobin actually produced.

It is not difficult to dissociate the iron from other potent factors (organic) in a variety of tissues. For example various fractions have been produced from liver, spleen, heart, and kidney which contained very small amounts of iron and yet showed much capacity to cause regeneration of new hemoglobin in anemic dogs ((12) see Table 5).

Liver ash (13) when fed to the anemic dog will produce about one-half as much hemoglobin as does the whole fresh liver. It would seem that the other half of the liver potency resided in the organic fraction.

Sturgis and Farrar (19) have shown that the salmon bread diet used in this laboratory (21) and the Cowgill diet (1) contained iron which was 50 per cent available by dipyridyl analysis. The Cowgill diet contains less iron but produces much more new hemoglobin in anemic dogs.

Even if iron were the only limiting factor involved in hemoglobin production in anemia our problem would not be simplified by the introduction of the term "available iron (dipyridyl)." The ultimate utilization of iron given orally is to a great degree governed by absorption. It is quite true that hematin iron is not absorbed to any great extent, if at all, but inorganic iron also is absorbed very poorly. Therefore, we have gained nothing by complicating the issue in stating that because 100 per cent of the metal in one of the inorganic salts reacts with dipyridyl it is all available *for absorption* when we know that actually very little will be absorbed.

In addition to this we know that iron is *not* the only limiting factor in the treatment of anemia. One or more organic factors are involved as can readily be appreciated from a survey of the work referred to above. Some would argue from results obtained in studying nutritional anemia in rats that the efficacy of liver in treatment of secon-

dary anemias in general was directly proportional to the available iron and copper content (7). This may be true as regards the milk nutritional anemia in rats. Here a deficiency in copper and iron has been produced and, as such, would be expected to respond to iron and copper therapy. It has been pointed out that nearly every article of food contains copper, in amounts varying from 0.1 mg. per kg. in celery to 44 mg. per kg. in fresh calves' liver (11).

There has yet to be found any condition in which a human being has been shown to be copper deficient or even notably low in copper as regards tissue content. If anything, quite the reverse has been found. Even the poorest of human rations contain significant quantities of copper. The tendency is for copper to be increased in many diseases, among which are anemias of various forms (3, 5, 14, 15). We do not deny that very small amounts of copper play a part in internal metabolism. Evidence has been forthcoming to show that it may influence the interchange of iron in the body (2, 10). But it has not been demonstrated to be lacking in any condition *except nutritional anemia in rats*, and so cannot be considered in any way as a limiting factor in the anemia of dogs due to blood loss or in secondary anemias of human beings generally.

SUMMARY

In experimental anemia in dogs due to blood loss the term "available iron" as determined by the dipyridyl test has no physiological significance. Iron salts (100 per cent available by dipyridyl) given in optimum dose (560 mg. per 2 weeks) will cause a net production of 50 to 55 gm. hemoglobin above the control base line in anemic dogs. This means that an iron salt which is rated as 100 per cent available by the dipyridyl test is only 35 per cent *physiologically available.*

The term "available iron (dipyridyl)" simmers down to iron not in the form of hematin compounds. The absorption of this "available iron" is conditioned by a great variety of factors, many unknown at this time.

Iron is indeed an elusive sprite whose "availability" or comings and goings cannot be determined in dogs by dipyridyl—perhaps only in part by studies of absorption and excretion.

Liver contains "available iron (dipyridyl)" but also *organic* factors

influencing hemoglobin regeneration in anemia as *liver ash* contains only about 50 per cent the potency of the whole liver.

One can readily dissociate the iron from other potent factors in various tissues. Fractions of heart, liver, spleen, and kidney may contain very little iron yet cause much hemoglobin regeneration in anemic dogs.

No investigator has reported any condition of copper deficiency in man or dog. In fact, in anemias copper is usually above normal concentration in the liver. It is unlikely, therefore, that in experimental anemia in dogs and in the various anemias of man, any significance attaches to the intake of copper.

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THE HEMOLYTIC EFFECT OF INDOL IN DOGS FED NORMAL DIETS

By C. P. RHOADS, M.D., and W. HALSEY BARKER, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research*)

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Experiment has shown (1) that anemia of severe degree occurs when indol is fed to dogs maintained on a deficient diet, causative of canine black tongue. The same amount of indol causes either no anemia or a transient and mild anemia in animals taking a normal, mixed diet. Sufficiently large amounts of indol will cause severe anemia, however, even if a normal diet is fed, hence the hypersusceptibility of the animal fed the deficient diet is quantitative rather than absolute.

Three explanations of the anemia-producing effect of indol are possible. (*a*) It is destructive to erythrocytes *per se* and more so when the diet is deficient, or (*b*) it exerts a depressing effect upon the production of erythrocytes by the bone marrow, or (*c*) a destructive action takes place that is uninfluenced by the diet but regeneration is less active in the presence of a poor diet.

To obtain information concerning the first of these possibilities the excretion of bilirubin has been studied in animals fed normal diets with and without the administration of indol. Only in this way could a quantitative measurement of the factor of blood destruction be obtained.

The relation between the rate of excretion of bile pigment and the rate of destruction of erythrocytes has been discussed by Rous (2) and his coworkers as well as by Whipple (3). Whereas the technical methods for the measurement of bile pigment are not sufficiently accurate to allow precise analysis, nevertheless under constant experimental conditions, pronounced and sustained variations in the output of bilirubin can be taken as satisfactory evidence of an increased rate of destruction of blood. According to Broun (4) 1 gm. of bilirubin represents 1 gm. of hematin, and the hemoglobin molecule yields 4

per cent of hematin by weight. For a detailed discussion of the subject the original papers should be consulted.

Methods

Sterile biliary fistulae were prepared according to a somewhat modified technic of Rous and McMaster (5). The common bile duct was sectioned, the fundus of the gall bladder opened *in situ* and a No. 24 catheter sewn in place. The gall bladder was then invaginated over the catheter to make a tight joint. The catheter was led to the pelvis and joined there by a glass U tube to a rubber tube which led out through an oblique incision just below the costal margin. The bile was collected in a rubber balloon which could be drained by a side arm. Strict aseptic precautions were observed whenever the line was opened. The external apparatus was covered by a wicker basket covered by two fitted canvas jackets closed by talon fasteners.

If adequate precautions are taken, sterile bile can be collected for months before infection sets in. The animals eat well and maintain their weight. No animal was employed for experiment in which the bile was infected or in which there was evidence of liver insufficiency as shown by an increased bilirubin content of the blood serum.

The daily output of bilirubin was measured by the method of McMaster (6). To save time, collections of 2 or 3 days were frequently analyzed together. This procedure was justified since only average yields over considerable periods were compared. As can be seen from the charts there exists a moderate day-to-day variation in the yield of bile pigment. These are quite out of the range of the very pronounced changes which are taken as evidence of variation in the rate of hemolysis.

The technic described, though difficult and cumbersome, was selected as the only one which was suitable.

The dogs were kept in metabolism cages to eliminate the factor of exercise which is known to affect the output of bile pigment. The normal diet fed was a uniform mixture of cooked beef, bread, and dog biscuit. Certain experiments were made on animals which were wholly deprived of bile and others in which 50 cc. of dog bile were fed daily. In rare instances beef bile was substituted for dog bile. Furthermore, the feeding of this amount of bile was not reflected in an increased rate of excretion of bile pigment by the fistula, although larger amounts of fed bile have been shown to do so (5). In no instance was a pathologic manifestation referable to deprivation of bile observed.

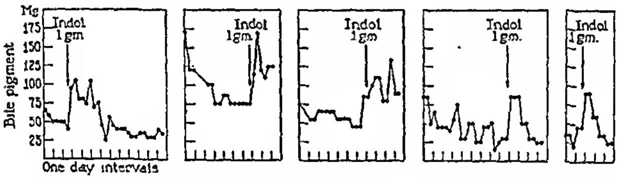
The indol was a commercial product of reasonable purity. It was fed by hand in ordinary absorbable gelatin capsules. The animals were observed carefully to see that the capsules were not vomited. A further check on absorption was at hand in the presence of indican in the urine.

Fistulae between the renal pelvis and the urinary bladder as well as simple determinations of the content of pigment in the stools and urine were discarded as unsatisfactory for these particular experiments.

RESULTS

The changes in the rate of excretion of bilirubin following the administration of indol during the feeding of a normal diet are shown in Text-fig. 1. In all of the experiments shown, a single dose of 1 gm. of indol was fed; a more sustained effect follows the repeated administration of the same amount. Such tests are open to the possible objection, however, that sufficient indol or its metabolic breakdown products are secreted in the bile to give a false colorimetric test for bilirubin

The increased rate of excretion of bilirubin is well defined, is greater than the spontaneous variations, and is sufficiently sustained to rule out technical error resulting from the effect of indol on the colorimetric determinations. Occasional instances are encountered in which no



TEXT-FIG. 1. Effect of the oral administration of indol on the rate of excretion of bilirubin.

increase can be detected and on the other hand rare instances have been encountered in which more marked increases are seen. The charts presented are typical of the average response.

DISCUSSION

From the experiments reported, it is quite apparent that when indol is administered to dogs taking a normal diet a slight and irregular increase of bilirubin output occurs. Since 1 mg. of bilirubin is derived from roughly 0.18 cc. of blood even an increased blood destruction of 9 cc. daily would result in an increased bile pigment output of 50 mg. or double the normal values. The remarkably labile blood volume of the dog and its apparently great reserve of blood in storage depots makes a loss of this amount of blood perfectly possible without the development of apparent anemia providing a normal diet is fed. The

studies of Robscheit-Robbins, Walden, and Whipple (7) present striking evidence of the amazing regenerative power for blood possessed by the normal organism. Hence an apparent failure of anemia to develop when indol is administered does not necessarily mean that indol is entirely without hemolytic power under normal dietary conditions. It simply indicates that sufficient hemolysis has not taken place to effect a discernible change in the number of circulating formed elements. This factor could of course be controlled by accurate measurements of the blood volume. Two objections to this procedure existed in our experiments. Sufficient blood for repeated blood volume determinations would represent a vastly greater loss than was reflected in the increased output of bilirubin. Secondly the available methods for determinations of blood volume are not accurate within the required limits.

Application of the method of Harrop and Barron (8) to the study of the liver function of the animals with total biliary fistulae indicated that not infrequently a distinct depression of function was present, even though no clinical jaundice was apparent. It is possible that the hemolytic effect of indol administration may reflect a slight though definite hypersusceptibility to that compound associated with the mildly abnormal experimental conditions. Certainly the drug was not as well tolerated as was the case in normal animals without biliary fistulae. Despite the oral administration of fresh dog bile the constant, slow delivery of bile from the common duct which exists normally could not be duplicated in the experiments.

The objection may be advanced that sufficient indol or its derivatives may have been excreted in the bile to give a false reading for bilirubin. Opposed to this is the fact that no indol could be demonstrated in the bile in more than a trace by steam distillation followed by spectroscopic study. Furthermore, it is clear from the figures that the increased output of bilirubin persisted for several days after a single dose of indol. Examination of the blood of the animals showed that normal indican levels were present 8 hours after the administration of 1 gm. of indol by mouth.

CONCLUSION

Indol is mildly hemolytic when fed to dogs taking normal diets.

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INDUCED SUSCEPTIBILITY OF THE BLOOD TO INDOL

By C. P. RHOADS, M.D., and D. K. MILLER, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research*)

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Studies previously reported from this laboratory (1) present evidence that the oral administration of amidopyrine is followed by anemia in dogs fed a deficient diet which is causative of black tongue. Neither amidopyrine alone, in the amounts employed, nor the diet alone, resulted in anemia under the experimental conditions observed. The anemia could be cured, moreover, by supplementing the diet with a factor in which it is deficient, even though the administration of amidopyrine was continued. This phenomenon of an increased susceptibility of the blood to amidopyrine under conditions of dietary deficiency, suggested the existence of a general principle which might also apply to the aromatic compounds produced by endogenous metabolism. Of those compounds, indol has been selected for study since some information is available concerning its metabolism.

Indol has been much studied chemically but relatively little as concerns the pathological effects which result from its administration. Houssay (2) has reported studies of the conjugation of indol and has reviewed the literature on the subject. Houssay concludes that indol is converted into indican by the liver, since that conversion occurs normally after removal of the intestine and after nephrectomy, but not after hepatectomy. Excretion by the kidney is proved by the presence of indican in the urine and by its accumulation in the blood after the removal of both kidneys. Büngeler (3) injected indol into mice and observed that anemia resulted, but since he was concerned only with the development of leukemia, the studies of the erythrocytes are not sufficiently detailed to be analyzed. Furthermore any procedure involving the injection of indol is not applicable, since unconjugated indol is not present in the circulating blood under any but the most abnormal circumstances (Houssay, 2). Certain facts exist, however, which suggest that the aromatic compounds derived from endogenous sources may play some rôle in causing disease. Tönnis and Horster (4) in a series of papers have described the production of indicanuria and anemia in dogs with surgically formed, inactive, open jejunal segments. Relief of anemia as well as the associated symptoms seems to have followed the administration of liver extract in these animals.

Methods

The animals employed were mongrel dogs of about 7 kilos in average weight. They were kept under standard conditions in individual cages with bedding of wood shavings.

The so called normal diet was one which is fed as a routine and empirically is known to be capable of maintaining dogs in good health over a period of several years. It is a mixture of cooked beef, bread, and dog biscuit. The black tongue diet is that described by Goldberger (5). It is known to cause acute black tongue when fed, without supplement, for a period of from 5 to 12 weeks. In an extensive study the feeding of this diet has never been known to cause symptoms in normal dogs after a shorter interval. The corn meal, peas, and casein were mixed and cooked for 2 hours in a steam cooker. The remaining ingredients were then added and thoroughly mixed. The animals were fed daily and were allowed to eat as much as they chose.

Blood was taken from the jugular vein in a standard amount of potassium oxalate for routine examinations. Determinations of the numbers of erythrocytes and leukocytes were made in standard pipettes and counting chambers. The hemoglobin was estimated by the Sahli method, employing a glass standard. The Sahli tubes were carefully calibrated and checked at frequent intervals by the O_2-combining capacity method of Van Slyke.

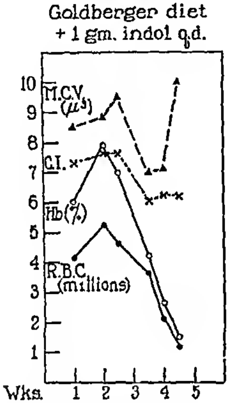
The indol used was a commercial crystalline product of reasonable purity. It was fed in ordinary absorbable capsules which were placed in the back of the animal's pharynx and forced with the finger to a point where they were swallowed. The animals were carefully observed to see that no capsules were regurgitated. The liver extract used was the powdered material (Eli Lilly and Company) of which 4 gm. are derived from 100 gm. of liver. It was made up to a 50 per cent solution in water and fed by stomach tube. Reduced iron (ferrum reductum U.S.P.) was used in a few experiments. It was administered in absorbable capsules containing 1 gm. each. The vegex was the commercial salt autolysate of brewers' yeast made up to a 50 per cent solution in water.

EXPERIMENTAL

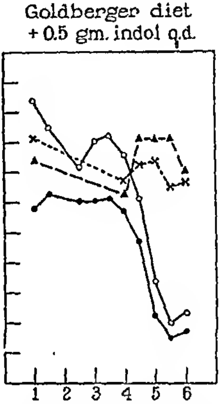
In all, 11 different types of experiments were made as follows: Indol was administered: (1) while a normal diet was fed; (2) as a brief experiment to dogs in a state of deficiency following the feeding of the black tongue diet; (3) during periods of deficiency and during subsequent periods when the diets were supplemented with yeast; (4) throughout successive periods of normal and deficient diets; (5) during periods of deficiency and during subsequent periods when the diet was supplemented with liver extract; (6) while an exclusive diet of milk was fed; (7) while a basal diet was fed which is not causative of

black tongue; (8) while the animals voluntarily abstained from food. (9) The effect on liver function of the administration of indol was tested. (10) The effect of splenectomy on the anemia-producing effect of indol was observed. (11) The levels of indol and indican in the blood were observed.

In this communication certain of the experiments are reported together for the sake of brevity.



TEXT-FIG. 1. Dog 1



TEXT-FIG. 2. Dog 2

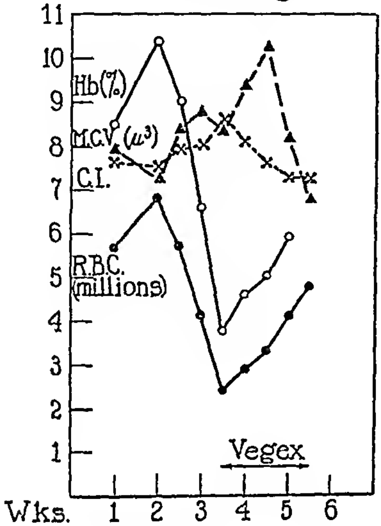
TEXT-FIGS. 1 to 9. Levels of the blood in 9 dogs given 1 gm. of indol daily after deficiency was established.

Acute Hypersusceptibility to Indol

Experiments 1 and 2 (Text-Figs. 1 to 9).—The black tongue diet was fed until the first signs of erythema of the labial mucous membrane appeared. This period varied from 6 to 10 weeks in different experimental animals. Indol was then fed in amounts of roughly 100 mg. per kilo of body weight. A prompt and very marked fall in the numbers of erythrocytes occurred, a decrease from 5,000,000 to something over 1,000,000 cells per mm.[3] of blood in less than 1 week being common. The anemia was associated with a normal or moderately

elevated leukocyte count and distinctly increased numbers of platelets. The animals became pale and lethargic but continued to take food in most instances. Examination of films of the blood revealed a striking

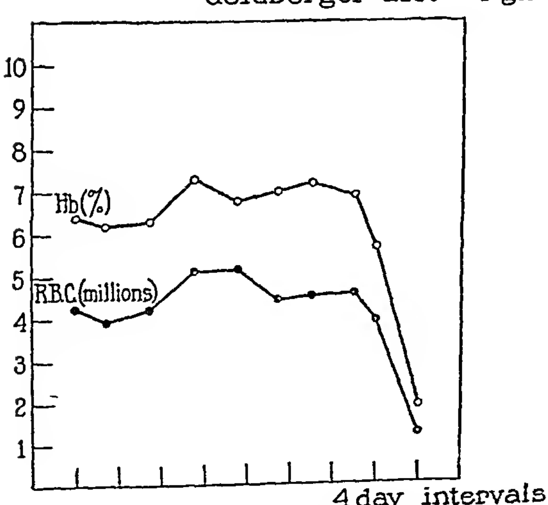
Goldberger diet + 1 gm. indol q.d.



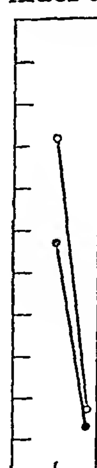
TEXT-FIG. 3. Dog 3

TEXT-FIG. 4. Dog 4

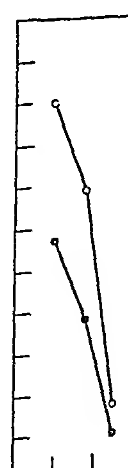
Goldberger diet + 1 gm. indol q.d.



TEXT-FIG. 5. Dog 5



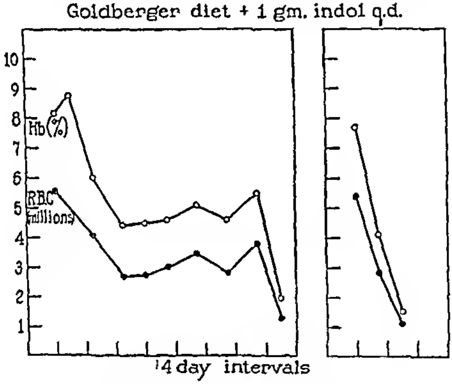
TEXT-FIG. 6
Dog 6



TEXT-FIG. 7
Dog 7

variation in the size and shape of the erythrocytes. If the administration of indol was continued the anemia occasionally terminated fatally but not infrequently an incomplete remission took place, marked by an increase of the circulating reticulocytes to levels between 10 and 20 per cent and an increase in the number of erythrocytes to between 2,000,000 and 3,000,000 per mm.³

Control animals taking a normal diet and fed the same amount of indol as those just described showed in most instances no decrease in erythrocyte levels greater than the variations which are normal for the dog. Occasionally a well defined drop in erythrocytes to levels



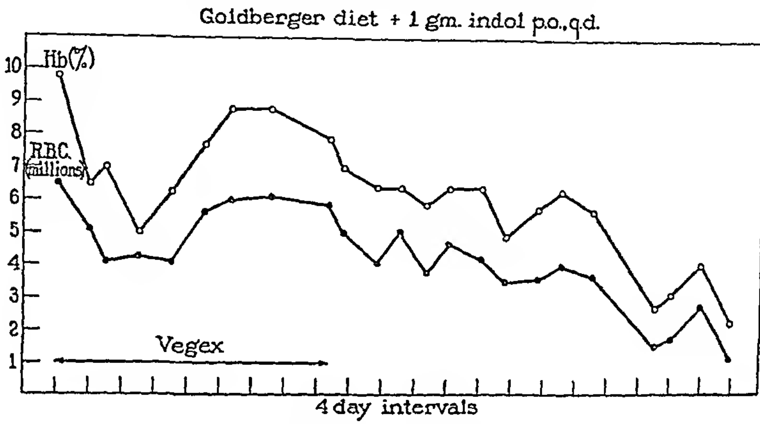
TEXT-FIG. 8. DOG 8 TEXT-FIG. 9. DOG 9

in the 3,000,000 per mm.³ range occurred, but this was distinctly unusual and was promptly recovered from, although the drug was continued. In no instance did any animal eating the normal diet show a degree of anemia which approached that regularly obtained in the animals fed the deficient diet.

Chronic Hypersusceptibility to Indol and the Treatment of the Anemia with Yeast

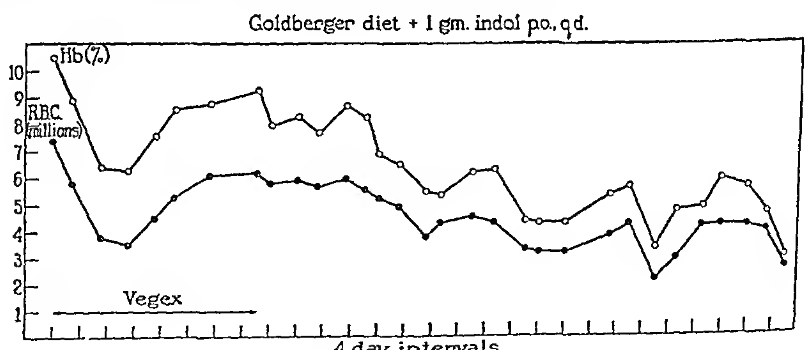
Experiment 3 (Text-Figs. 10 and 11).—Previous studies had shown that the symptoms following regularly the feeding of the black tongue

diet without supplement could be prevented by the administration of yeast rich in the vitamin B_2 (G) complex, Goldberger (5). Accordingly the effect of supplementing the diet with a suitable yeast source of that vitamin was tried in animals with anemia resulting from the



TEXT-FIG. 10. Dog 10

TEXT-FIGS. 10 and 11. Levels of the blood in 2 dogs given 1 gm. of indol daily during periods of yeast supplement to the black tongue diet and during subsequent periods of yeast when supplement was omitted.



TEXT-FIG. 11. Dog 11

administration of indol during a state of deficiency. The results are shown in Text-figs. 10 and 11. Following the supplement with yeast a prompt and decided rise in the levels of the blood took place which could be maintained as long as the supplement was given, although the administration of indol was continued throughout the

experiment. When the supplement was discontinued, however, progressive anemia appeared.

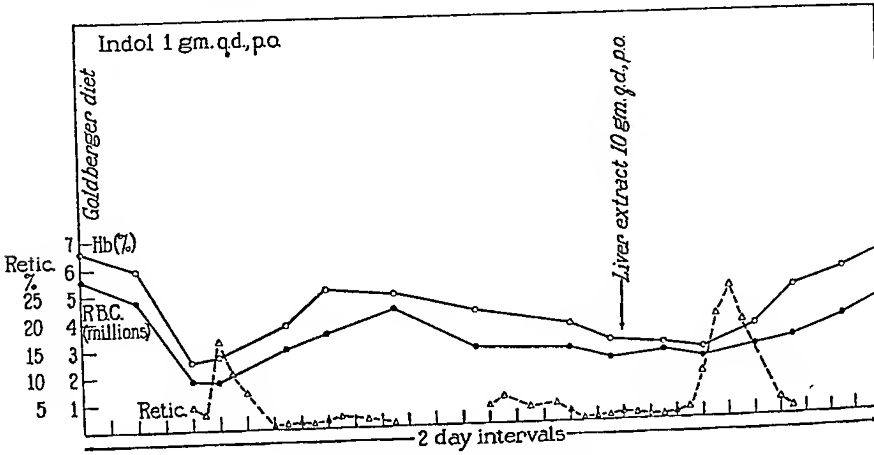
Chronic Hypersusceptibility to Indol and the Treatment of the Anemia with Liver Extract

Experiments 4 and 5 (Text-Figs. 12 to 18).—Goldberger (6) has shown that liver extract (Lilly N.N.R.) is preventive of the symptoms of black tongue which resulted from the feeding of the diet used in our experiments. Since the same liver extract is also preventive and curative of pernicious anemia in human beings the effect of its administration was studied in the chronic anemia in the dog resulting from indol and the deficient diet.

In three animals, Nos. 12, 13, and 14, Text-figs. 12, 13, and 14, the black tongue diet was fed until a definite state of deficiency was considered to have been established. Indol was then administered as in Experiment 1 and the anemia promptly developed. Liver extract (Lilly N.N.R.) was then fed by stomach tube in 10 gm. amounts daily. In every instance an increase in the number of circulating erythrocytes took place, associated with an increase in the levels of hemoglobin. The supplement of liver extract was discontinued as soon as approximately normal levels of the blood were obtained, but the feeding of indol as well as the deficient diet was continued.

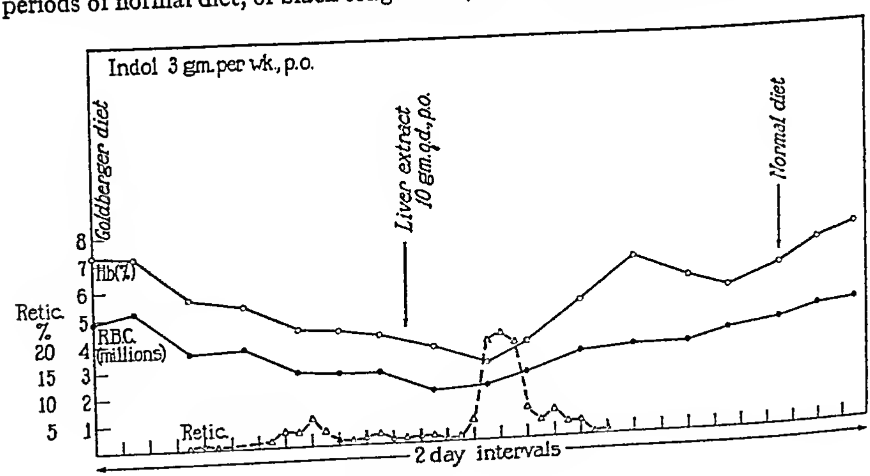
As would be expected from the results of the short experiments, after an interval of from 3 to 5 weeks from the time when the supplement of liver extract was omitted, a slow, progressive decrease in the levels of erythrocytes and hemoglobin appeared and continued until only between 2,000,000 and 2,500,000 erythrocytes per mm.^{3} were present. This was considered to be a suitably severe form of anemia for test. The levels of reticulocytes varied somewhat during the periods of the anemia. In certain instances they were irregularly elevated for a time and in others no elevation above 2 per cent appeared. In every experiment the reticulocytes were allowed to become stabilized at low levels before therapeutic test. When a severe anemia, with reticulocytes stabilized at a low level, had been obtained, liver extract 10 gm. (Lilly N.N.R.) was administered daily by stomach tube. In every animal a prompt and decided rise of reticulocytes occurred. Levels of from 10 per cent to 75 per cent were obtained within 10 days after

treatment was begun. The number of reticulocytes promptly fell and this was followed by a rise in the levels of erythrocytes and hemo-



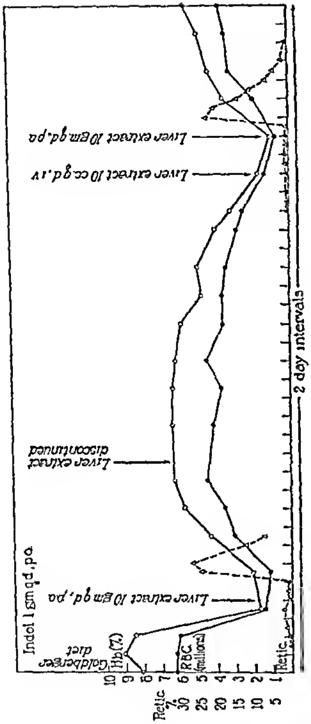
TEXT-FIG. 12. Dog 12

TEXT-FIGS. 12 to 18. Levels of the blood in 7 dogs given indol daily during periods of normal diet, of black tongue diet, and of supplement with liver extract.

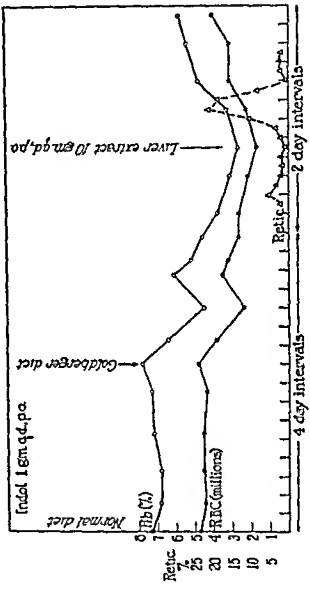


TEXT-FIG. 13. Dog 13

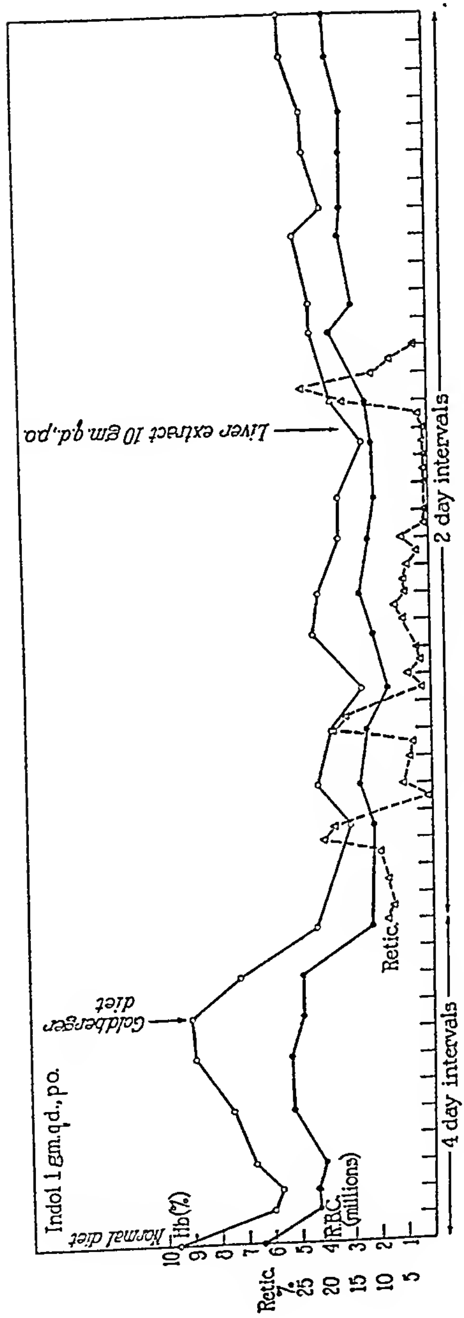
globin. Simple supplement of the diet with liver extract was not sufficient to restore absolutely normal values for the blood in every experiment, and where this was the case a normal diet was substituted



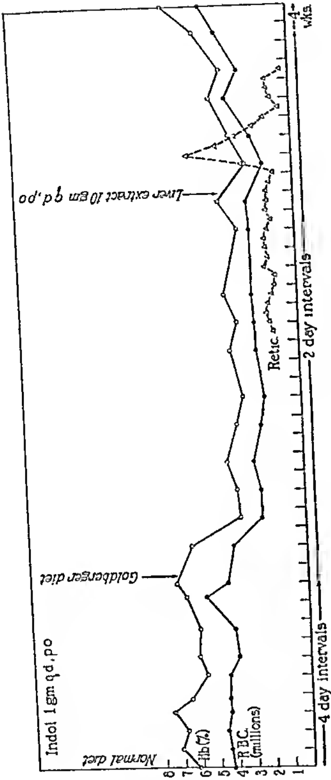
TEXT-FIG. 14. Dog 14



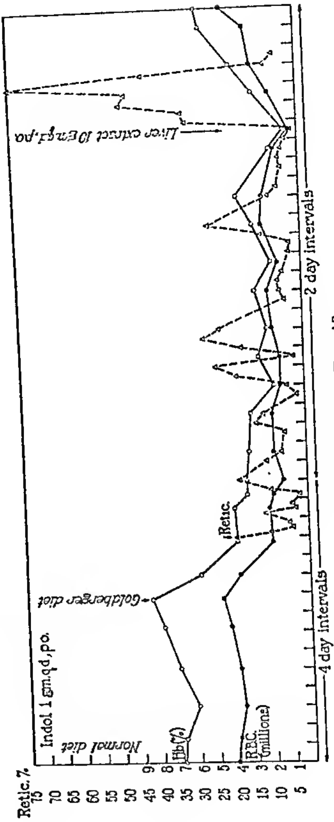
TEXT-FIG. 15. Dog 15



TEXT-FIG. 16. Dog 16



TEXT-FIG. 17. Dog 17



TEXT-FIG. 18. Dog 18

for the deficient one. This move effected complete cure of anemia in every instance despite the continued feeding of indol.

In the greater number of the observations, Nos. 15 to 18, a control period was observed, during which the normal diet and a standard amount of indol were fed. In some instances, as in animals 15 and 17 there was no fall of blood levels but occasionally, as in observations 16 and 18 a mild decrease of erythrocytes and hemoglobin occurred which promptly disappeared as soon as sufficient time had elapsed to allow the normal hematopoietic mechanism to accommodate for the increased demand put upon it by the administration of indol. After the normal diet had been fed sufficiently long to establish the fact that the indol was tolerated without anemia, the black tongue diet was substituted and the administration of indol was continued. As in the experiments just described a gradual fall of the numbers of erythrocytes and of hemoglobin resulted until a severe degree of anemia was established. As before, a considerable variation in the numbers of reticulocytes was observed in the first part of the period of anemia but in due time they became stabilized at low levels. The institution of therapy with liver extract, other factors remaining unchanged, was followed by the expected reticulocyte peak and by an increase of blood values to levels which approached the normal. A striking feature of the response to the administration of liver extract was an increase in the number of leukocytes, although they had not been notably few during the severe phase of anemia. This increase usually preceded the increase in erythrocyte numbers by several days and coincided with the appearance of elevated levels of reticulocytes.

The histological changes in the bone marrow will be described in a separate communication. It suffices to state here that biopsies of femoral marrow, removed during a control period before the experiment was begun, showed a normal distribution and the expected orderly maturation of cells with all stages of development represented. Specimens of femoral marrow removed by biopsy and at autopsy at the height of the anemia showed a very marked increase in the cellularity of the marrow with a lack of mature forms, both of the red and white cell series. Biopsies of marrow after weeks of the administration of indol with a normal diet showed no significant variation from normal.

Protocols of Experiments 4 and 5.—

Animal 12.—

Dec. 22. Goldberger diet begun. Weight 17.5 kilos.

Jan. 22. Indol feeding begun, 6 gm. weekly. R.B.C. 5,500,000. Hb 66 per cent. W.B.C. 15,000.

Jan. 29. Very pale and weak. No stomatitis. R.B.C. 1,830,000. Hb 25 per cent. W.B.C. 51,500. Reticulocytes 4.2 per cent.

Jan. 31. General condition improved. R.B.C. 1,760,000. Hb 27 per cent. W.B.C. 25,200. Retic. 16.2 per cent. Despite the continuation of the feeding of the diet and the administration of indol a mild remission occurred, presumably explained by the extension of active, hematopoietic marrow.

Feb. 13. General condition excellent. R.B.C. 4,330,000. Hb 48 per cent. W.B.C. 10,000. Retic. 0.8 per cent. From this point a slow fall of the levels of the blood took place, possibly because no further extension of hematopoiesis was possible under the experimental conditions.

Mar. 1. Animal very pale and weak. No stomatitis. R.B.C. 2,360,000. Hb 30 per cent. W.B.C. 10,500. Retic. 0.8 per cent. Therapy with liver extract begun, 10 gm. daily, by mouth.

Mar. 8. General condition improved. R.B.C. 2,229,000. Hb 26 per cent. W.B.C. 74,800. Retic. 8.0 per cent. The marked elevation of white count and beginning of a rise of reticulocytes indicates the onset of remission.

Mar. 15. General condition excellent. R.B.C. 2,910,000. Hb 48 per cent. W.B.C. 19,500. Retic. 1.4 per cent. The rise of reticulocytes has subsided and an increase of levels of the blood is in progress.

May 9. General condition excellent. R.B.C. 5,150,000. Hb 82 per cent. W.B.C. 20,500.

Animal 13.—

Jan. 25. Feeding of Goldberger diet begun. Weight 8.6 kilos. Administration of indol begun, 3 gm. weekly. R.B.C. 5,140,000. Hb 72 per cent. W.B.C. 10,300. Retic. 0.4 per cent.

Feb. 15. Pale and weak but eating well. R.B.C. 1,990,000. Hb 36 per cent. W.B.C. 11,600. Retic. 1.2 per cent. Treatment with liver extract, 10 gm. daily by mouth, was begun.

Feb. 19. General condition slightly improved. R.B.C. 2,050,000. Hb 29 per cent. W.B.C. 42,600. Retic. 18.8 per cent. The elevated leukocyte and reticulocyte counts indicate the impending remission.

Mar. 1. General condition excellent. R.B.C. 3,370,000. Hb 66 per cent. W.B.C. 13,000. Retic. 1.4 per cent. The numbers of leukocytes and reticulocytes have become normal and the increased levels of the blood are clearly in evidence.

Mar. 12. Normal diet substituted for the Goldberger diet. R.B.C. 4,000,000. Hb 61 per cent. W.B.C. 13,300.

Mar. 19. Condition excellent. Experiment terminated. R.B.C. 4,560,000.

Hb 74 per cent. W.B.C. 12,100. In a second experiment with similar course this animal was destroyed at the height of the anemia and the bone marrow subjected to histological study.

Animal 14.—

Nov. 30. Feeding of Goldberger diet begun. Weight 10.5 kilos.

Jan. 2. Administration of indol begun, 6 gm. weekly. Animal in excellent condition. R.B.C. 6,250,000. Hb 91 per cent. W.B.C. 11,500. Retic. 0.4 per cent.

Jan. 7. Animal very pale and weak; slight erosion of labial mucous membranes. Eating diet. R.B.C. 1,430,000. Hb 17 per cent. W.B.C. 23,100. Retic. 2.2 per cent. Supplement of liver extract, 10 gm. daily, by mouth begun.

Jan. 11. Animal in poor condition; not eating the diet. R.B.C. 1,150,000. Hb 21 per cent. W.B.C. 76,200. Retic. 24.6 per cent. This was the first day of the change in the number of leukocytes and reticulocytes as an indication of the beginning of the induced remission.

Jan. 23. Animal in excellent condition. Eating well. Liver extract discontinued. R.B.C. 4,540,000. Hb 63 per cent. W.B.C. 10,000. Retic. 0.8 per cent. From this point on there was a prolonged slow drop in the levels of the blood.

Feb. 8. Superficial early erosions of the labial mucous membranes have appeared. R.B.C. 3,680,000. Hb 58 per cent. W.B.C. 11,900. Retic. 2.4 per cent.

Feb. 25. Ulcerated lesions of the tongue and cheeks are well defined. Animal weak and pale. Not eating. R.B.C. 1,370,000. Hb 17 per cent. W.B.C. 21,800. Retic. 1.2 per cent. Liver extract, 10 cc. intravenously, daily.

Mar. 1. Animal very weak and pale. R.B.C. 750,000. Hb 10 per cent. W.B.C. 139,900. Retic. 0.8 per cent. The anemia was so severe that it was supposed that the animal's life was in danger in spite of the intravenously administered liver extract. Actually in light of subsequent observations the rising leukocyte count and the drop of general blood levels should have been taken as an indication of the onset of remission. Therapy was changed to orally administered liver extract however.

Mar. 3. The reticulocyte count has risen today to 22.8 per cent and the animal is markedly improved. Since the peak of reticulocytes has occurred only 48 hours after the institution of oral therapy, it is quite clear that it is in reality due to the parenterally administered substance.

Mar. 5. General condition excellent. Animal eating well. R.B.C. 1,880,000. Hb 35 per cent. W.B.C. 6,800. Retic. 13.6 per cent.

Mar. 12. Animal apparently well. R.B.C. 3,360,000. Hb 48 per cent. W.B.C. 10,900. Retic. 0.4 per cent. The peak of reticulocytes is well past and a clear remission of the anemia is in progress.

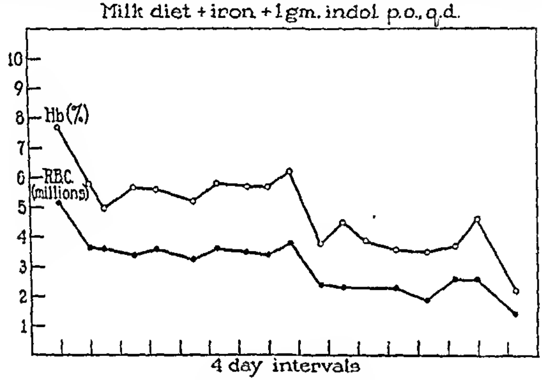
Apr. 30. General condition excellent. R.B.C. 3,860,000. Hb 53 per cent. W.B.C. 11,400. As was so frequently the case in these studies, liver extract,

though completely effective in causing remission, rarely enabled the animal to attain a completely normal level of the blood. Accordingly a normal diet was substituted for the Goldberger diet, the administration of indol being continued.

May 21. Normal levels of blood have been obtained. Experiment discontinued. R.B.C. 5,150,000. Hb 88 per cent. W.B.C. 11,000.

Hypersusceptibility to Indol Resulting from Diets of Milk

Experiment 6 (Text-Figs. 19 to 21).—All the studies of deficiency disease which have involved the use of the Goldberger diet causing

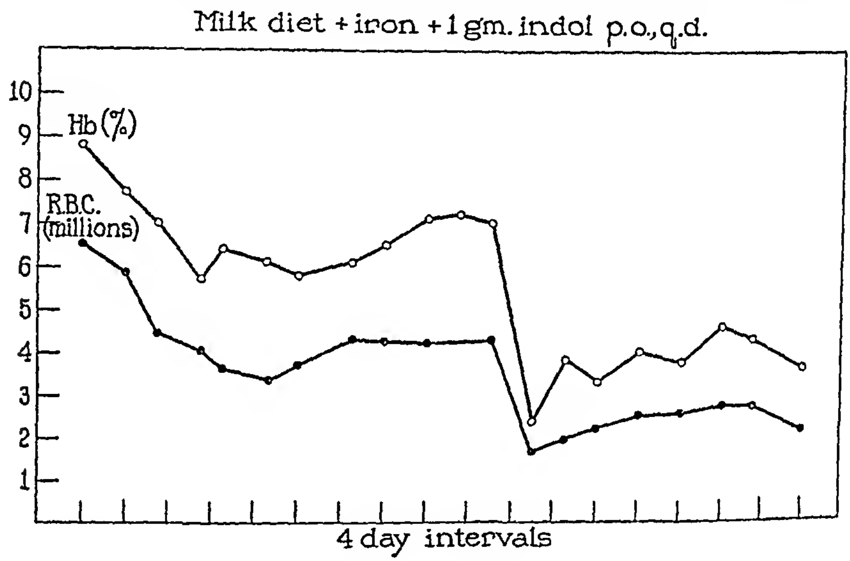


TEXT-FIG. 19. Dog 19

TEXT-FIGS. 19 to 21. Levels of the blood in 3 dogs given indol daily while fed a diet of milk with and without supplement by liver extract.

black tongue have been open to certain objections. Among them is the fact that the clinical syndrome of black tongue does not occur when the simple diet of Cowgill, which lacks the heat stable fraction of the vitamin B complex, is fed (Zimmerman, 7). Moreover, the Goldberger diet is exceedingly high in its content of corn and throughout the literature of pellagra there runs a strong suggestion that corn has some peculiarly specific action in causing stomatitis and central nervous system lesions. Rhoads and Miller (8) have shown that the Goldberger diet is not free of the heat stable component of the vitamin

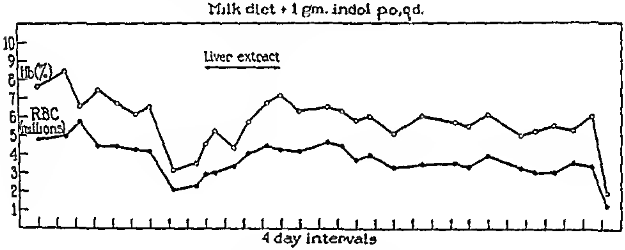
B complex which is required for growth in rats, since rats fed that diet grow at a normal rate. Certain other species, notably guinea pigs and swine, however, will not survive when fed the diet unless it is supplemented with liver extract (9, 10). Evidence has been advanced recently by Birch, György, and Harris (11) as well as by Street (12) that black tongue can be produced by feeding simple basal casein, fat, and glucose diets supplemented with crystalline vitamin B_1 and pure flavin (B_2). In view of the slight doubt concerning the cause of the peculiar syndrome which follows the feeding of the Goldberger



TEXT-FIG. 20. Dog 20

diet, however, it was necessary to control the experiments which have been described by combining the administration of indol with some diet which contained no corn. In a series of unrelated experiments dogs were fed a diet composed exclusively of milk (200 cc. per kilo of body weight daily). To our surprise the animals developed characteristic stomatitis of black tongue and died after a period of about 2 months. Here then was a diet which was known to be rich in flavin, and which was presumed to be rich in the remainder of the vitamin B_2 complex, but which caused black tongue. The objections which could be raised to the experiments on corn diets could be avoided by

feeding milk. Furthermore, although chronic black tongue, produced by treating insufficiently the acute phase of the disease, will result in anemia irregularly, the feeding of milk alone to adult dogs has never done so in our experience. Accordingly a series of animals were fed an exclusive diet of milk and were given the usual dose of 100 mg. per kilo of indol daily, reduced iron 1 gm. daily was administered as a supplement (Text-figs. 19 to 21). Exactly the same anemia occurred as was the case when the Goldberger diet was fed. Severe anemia developed which could be cured by feeding a normal diet or by supplementing the diet of milk by liver extract even though the administration of indol was continued.



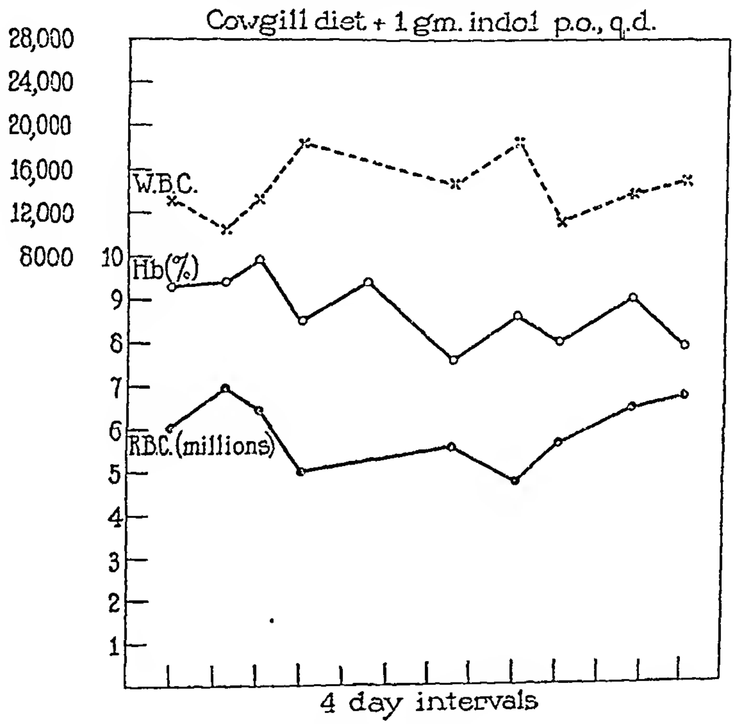
TEXT-FIG. 21. Dog 21

Failure to Induce a Susceptibility of the Blood to Indol by Feeding a Basal Diet

Experiment 7 (Text-Figs. 22 and 23).—To control further the dietary factors involved in the experiments, animals were fed the basal diet described by Cowgill (7), and indol was administered at the same time. Both factors of the vitamin B complex were omitted from the diet in the hope that it would be possible to establish the susceptibility of the blood to indol as being due to a lack of some part of that complex. The results were somewhat irregular but in general the indol was tolerated without severe anemia, quite contrary to the absolutely uniform anemia obtained by feeding indol with a Goldberger diet (Text-figs. 22 and 23).

This result was unexpected to some extent and no well substantiated

explanation can be advanced at this time. It is possible, however, that the very high content of casein of this diet may furnish a sufficient amount of the anti-black tongue factor to prevent the susceptibility to indol. It is striking that although the diet is supposedly a basal ration it never gives rise to the symptoms of black tongue. Further studies of this question are in progress, since possibly the rations of Birch, György, and Harris (11), or of Street (12), containing less



TEXT-FIG. 22. Dog 22

TEXT-FIGS. 22 and 23. Levels of the blood in 2 dogs given indol daily while fed the basal ration of Cowgill.

casein and causing black tongue would cause the hypersusceptibility to develop.

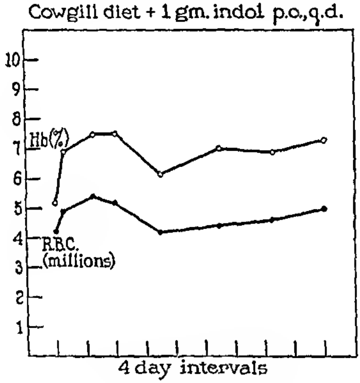
Susceptibility of the Blood to Indol Following Voluntary Abstention from Food

Experiment 8 (Text-Fig. 24).—The effect of dietary on the tolerance of the blood to indol is well shown in this experiment. Fig. 24 shows

the course of the anemia in one such animal. Although the black tongue diet was offered each day the food was completely refused. A severe anemia developed promptly, in sharp contrast to the result following the administration of the same amount of indol when a normal diet is taken.

Liver Function Not Affected by Indol

Experiment 9.—Experiments have been published by Miller and Rhoads (13) which show that liver function, as measured by the power of that organ to excrete bilirubin intravenously injected, decreases in

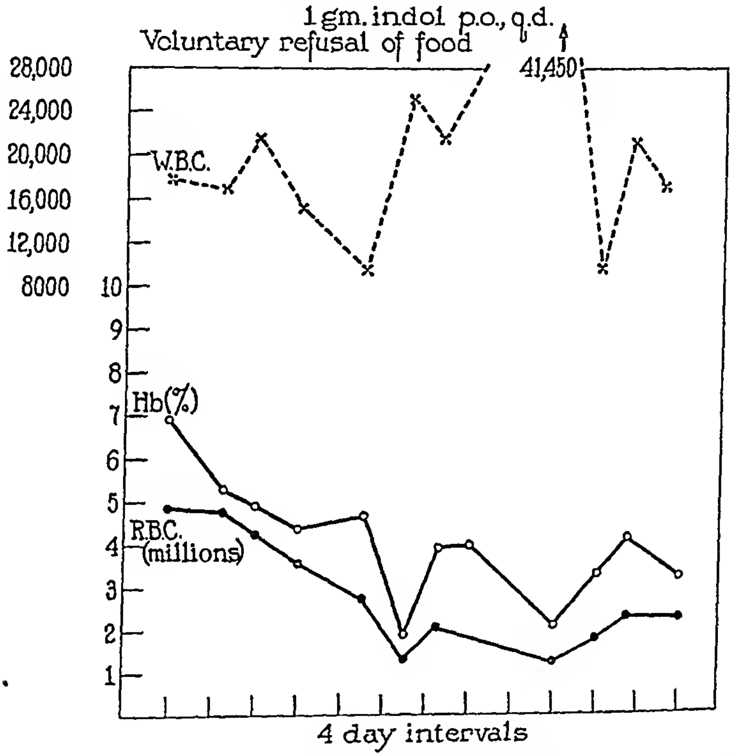


TEXT-FIG. 23. Dog 23

the deficiency which results from feeding the black tongue diet. This finding of deranged liver function is in accord, furthermore, with the beneficial effect of therapy with liver extract. It is required to show, however, in a control experiment that indol *per se* does no damage to liver function. Accordingly the experiment was made of administering 1 gm. of indol daily to 2 dogs taking a normal diet and following the power of the livers to excrete bilirubin. No change was seen in this function over a period of 1 month and no anemia developed. It was concluded that indol did not injure the hepatic function under the experimental conditions observed.

The Effect of Splenectomy

Experiment 10.—In view of the therapeutic effect of splenectomy on certain hemolytic anemias in human beings it was of importance to ascertain whether or not the operation would prevent the development of indol anemia. Accordingly splenectomy was performed in 2 dogs and complete recovery was allowed to occur. The deficient diet was



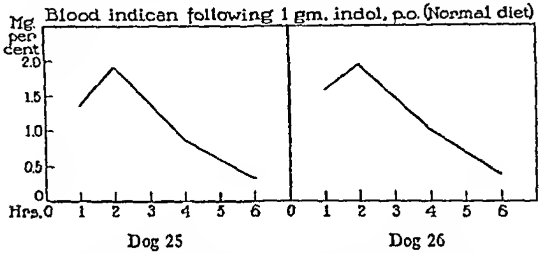
TEXT-FIG. 24. Dog. 24. Levels of the blood in a dog given indol while voluntarily abstaining from food.

then fed and after 4 weeks indol was administered. Anemia of the same degree as that seen in the non-splenectomized animals appeared at the usual time.

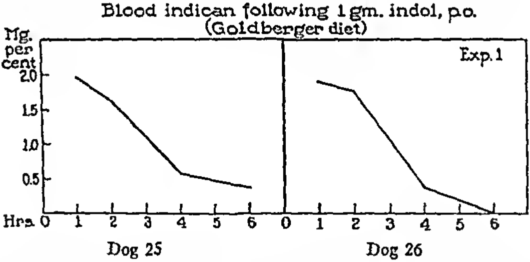
The Levels of Indol and Indican in the Blood

Experiment 11 (Text-Figs. 25 and 26).—The pronounced difference observed between the susceptibilities to indol of normal and deficient

animals suggested that the deficient state resulted either in some change of the rate of absorption of indol from the intestinal tract or of its conversion to indican. These possibilities were subjected to experimental test. By the method of Sharlit (14) the levels of indican in the blood following the oral administration of 1 gm. of indol were ascer-



TEXT-FIG. 25. Levels of indican in the blood at hourly intervals after the oral administration of 1 gm. of indol during the feeding of a normal diet.



TEXT-FIG. 26. Levels of indican in the blood at hourly intervals after the oral administration of 1 gm. of indol during the feeding of a black tongue diet.

tained in two dogs fed a normal diet, and in the same 2 animals after a well defined state of deficiency had resulted from feeding the black tongue diet. As shown in Text-figs. 25 and 26 there was no change detectable between the two nutritional states. Furthermore the levels of indol in the blood were ascertained by the method of Mazzocco (15) in parallel with the levels of indican following both the oral and intra-

venous administration of indol. No difference could be seen between the normally fed animals and those on deficient diets.

DISCUSSION

Certain factors in the experiments which have been described warrant discussion. As seen in the text-figures, the feeding of indol to dogs on a normal diet does result occasionally in a mild anemia which promptly disappears. Histological studies of the bone marrow during this phase of preliminary slight anemia show normal maturation of erythropoietic cells and some increase in number. It is quite apparent that indol feeding is a strain, though a mild one, on hematopoiesis in normally fed dogs and that it is readily compensated for by an increase of functional marrow. When the deficient diet is fed, however, a wholly different picture results from the administration of the same quantity of indol.

The rapid and profound drop in red cells strongly suggests an actual destructive process. In view of the known life of the red cell, anemia would develop much more gradually were only maturation interfered with. Proof of the hemolytic nature of the process will be presented in a subsequent communication.

From the histological appearance of the marrow it seems clear that a great increase in the number of immature marrow cells is a feature at the height of the anemia. Only two possibilities exist to explain this change: (*a*) that the deficient diet lacks a factor which is required for the maturation of erythropoietic cells, and (*b*) that all cells beyond the stage of erythroblast and myeloblast are destroyed in a hemolytic process which is stopped by something contained in liver extract. The early rises of reticulocytes indicate that the marrow can manufacture young cells, or, if the pure hemolytic theory be accepted, that not all reticulocytes are hemolyzed. According to Dock (16) an increase of reticulocytes is as much a feature of cessation of hemolysis as it is of the supplying of a specific maturation factor. From the evidence at hand the final answer cannot be obtained; subsequent communications will deal with this question in greater detail. It is entirely possible, indeed probable, that both processes play some rôle.

Sufficient information is not available from these experiments to discuss the question of oral *versus* parenteral therapy. Suggestive

evidence is at hand from Experiment 3 which favors the view that liver extract parenterally administered is effective. From the studies of Richter, Ivy, and Meyer (17) it is clear that the dog's stomach has little anti-anemia potency for the human heing, and this is also true for the liver of the dog. Castle (18) and his coworkers have shown that the anti-anemia principle in liver extract is dependent upon the interaction of a dietary constituent of normal gastric juice.

Since the dog has neither the gastric nor the liver factor we infer that in hematopoiesis it uses the dietary factor as such without the interaction with the gastric factor which is required in the human being. Helmer, Fouts, and Zerfas (19) have shown that liver extract is rich in the dietary factor and it is our belief that in the dosage employed this factor is the effective one in the dog experiments. Until more purified fractions for the treatment of pernicious anemia in the human being are available it seems unwise to continue experiments with liver extract parenterally injected. The results may always be open to the objection that sufficient dietary factor is present to produce an effect.

An analogy inevitably suggests itself between the experimental disease just described and pernicious anemia in the human being. The points of similarity are striking: mucous membrane lesions, gastro-intestinal disturbances, characteristic changes in the morphology of the erythrocytes, absence of hemorrhagic phenomena, low reticulocytes, and a characteristic response to the administration of liver extract. Certain well defined differences are also apparent. Macrocytosis is not a feature since as many or more microcytes are present in the smear than are macrocytes. No serious degree of indolemia or indicanuria can be demonstrated in pernicious anemia and although suppression of maturation in the bone marrow is a feature in the human being, the cell type which predominates in the marrow in these experiments on dogs cannot be proved to be the cell which is predominant in pernicious anemia. No claim is made that the experimental disease has any connection with any disease state of human beings.

The objection will be advanced to these experiments that in the studies reported by Rhoads and Miller (20) the occurrence of anemia in chronic black tongue produced by feeding a Goldberger diet alone has been described. In that publication it is specifically stated that

only after a prolonged chronic disease has been produced by treating insufficiently the acute phase does any anemia occur. Even then it occurs irregularly and is of mild degree. At no time has severe anemia been observed in this laboratory in experiments on a large number of animals during *acute* black tongue. Furthermore in the experiments presented here the controls with milk diets and the voluntary refusal of food rule out any specific effect of the black tongue diet, other than the deficiency.

It appears that under particular dietary circumstances the feeding of indol to dogs produces an effect which is not apparent when a normal diet is fed. Furthermore the effect may be caused to disappear by supplementing the diet with the factor in which it is deficient. Since neither the mode of action of the Goldberger diet, nor the active constituent of liver extract are known exactly it would be idle to speculate at this time concerning the exact mechanism by which the anemia is produced.

CONCLUSIONS

1. Indol, orally administered, causes anemia when certain deficient diets are fed.
2. The same amount of indol causes no considerable hematologic disturbance when normal diets are fed.
3. The anemia can be cured by supplementing the diet with liver extract, or by substituting a normal diet for the deficient diet.
4. Neither the diet alone nor the administration of indol alone produces marked anemia under the experimental conditions observed.

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THE INCREASED SUSCEPTIBILITY TO HEMOLYSIS BY INDOL IN DOGS FED DEFICIENT DIETS

By C. P. RHOADS, M.D., W. HALSEY BARKER, M.D., and D. K. MILLER, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research)

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Evidence has been presented in previous communications (1, 2) that indol, orally administered in suitable amounts, is hemolytic in dogs and that when the animals are fed deficient diets anemia results. The same amount of indol fed to dogs taking a normal diet has little or no effect in causing anemia. Since the hemolytic effect of indol has been proved, two possible ways exist in which anemia might result when the deficient diet is fed: (*a*) the hemolytic effect of the indol may be more marked in the presence of a deficiency or (*b*) the hemolytic effect may be constant and the bone marrow may be less capable of forming erythrocytes when the diet is unsuitable. To settle this question prolonged studies of the excretion of bile pigment and of the erythrocyte and hemoglobin levels in dogs receiving indol and fed normal diets, deficient diets, and deficient diets supplemented by the lacking factor have been made.

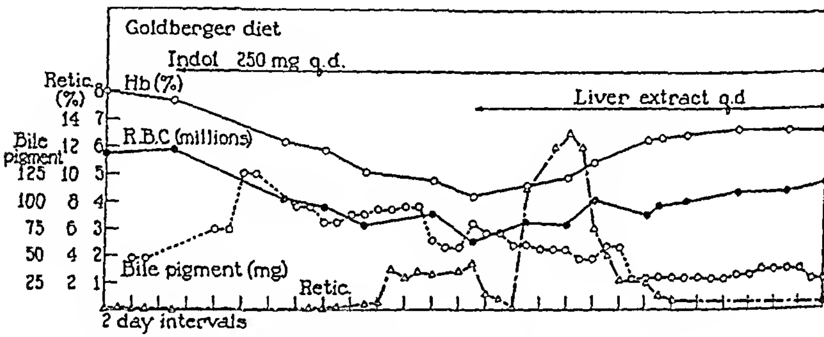
Methods

The technic employed for collecting the total bile in a sterile condition has been described (1, 2). It is a modification of that developed by Rous and McMaster (3). The measurements of the content of bilirubin in the bile have been made by the method of McMaster, Brown, and Rous (4). Although the method is open to certain theoretical objections the experimental results are valid since the interpretation depends upon the comparative values over different periods. The normal diet fed is a mixture of cooked beef, bread, and dog biscuit known empirically to be adequate in its content of vitamins. The Goldberger diet has been described repeatedly in previous publications (5), and is known to produce black tongue regularly in from 5 to 12 weeks. Since that effect can be prevented uniformly by feeding autoclaved yeast and other substances which are rich in their content of vitamin B_2 (G) and cannot be prevented by flavin (Koehn and Elvehjem, 6), its effect is supposed to be due to a lack of a part of the vitamin B_2 (G) complex other than the rat acrodynia factor or flavin.

Fresh dog bile was refed in 50 cc. amounts twice weekly to all the animals. Blood was taken at regular intervals from the jugular vein in standard amounts of potassium oxalate. Counts were made using standardized pipettes and counting chambers. The hemoglobin was estimated by the Sahli method, using calibrated tubes. The indol was a commercial crystalline product. It was fed by hand in ordinary absorbable capsules. All dogs were dewormed with hexylresorcinol and castor oil 1 month before they were put on experiment. The liver extract was Lilly (N.N.R.) powder, 4 gm. of which are derived from 100 gm. of liver. It was made up with water to a 25 per cent solution. In most instances the animals took it avidly.

EXPERIMENTAL

Experiment 1 (Text-Fig. 1).—This animal was fed the deficient diet for 4 weeks before the biliary fistula was formed. The output of bilirubin was allowed to



TEXT-FIG. 1. Dog 1

TEXT-FIGS. 1 to 7. Levels of reticulocytes, erythrocytes, hemoglobin, and daily excretion of bilirubin in dogs fed indol during periods of good and bad diets.

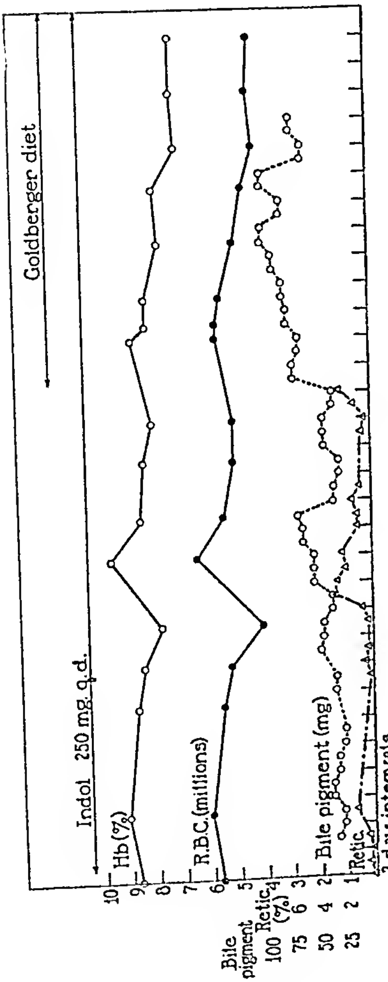
stabilize at about 50 mg. daily before indol was fed. Throughout the period depicted in the text-figure 250 mg. of indol were fed daily. 4 days after the first indol a daily output of bilirubin of 126 mg. was obtained which decreased slightly thereafter to stabilize at a figure between 80 and 90 mg. A progressive decrease in the blood levels from 5,780,000 erythrocytes and 80 per cent hemoglobin to 2,500,000 erythrocytes and 42 per cent hemoglobin took place without any significant elevation of reticulocytes. Over an 18 day period 1,568 mg. of bilirubin were excreted, an average of 87 mg. per day. The diet was then supplemented with 5 gm. of liver extract daily. On the 4th day the reticulocytes increased to 9 per cent and on the 7th day to 13 per cent followed by a progressively rising erythrocyte count to 4,360,000 and hemoglobin values to 63 per cent. Nothing was changed except for the addition of liver extract. The rate of excretion of bilirubin dropped sharply to 50 mg. daily, concurrently with the rise in numbers of reticulocytes, and then continued to decrease slowly. In the 18 days of treatment

859 mg. of bilirubin were excreted, an average of 47 mg. per day, a reduction of nearly 50 per cent of the untreated levels.

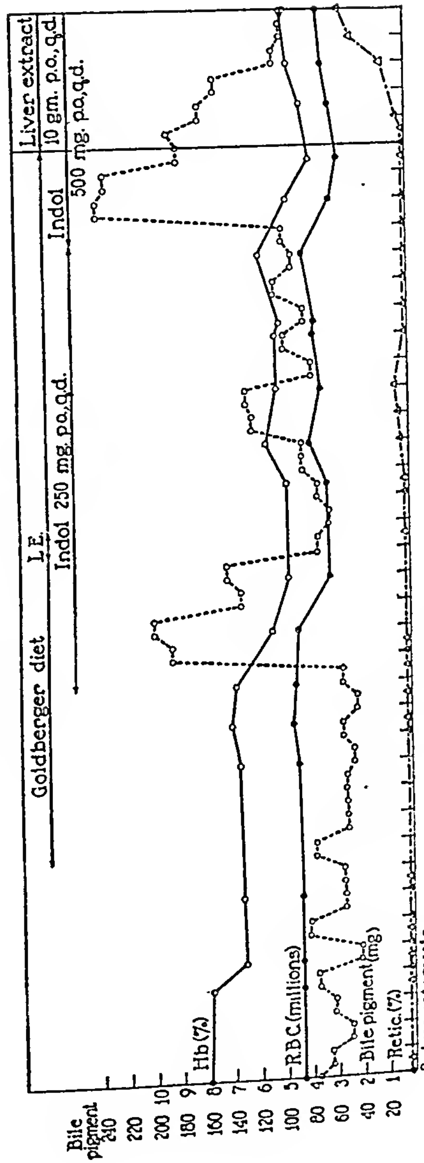
Experiment 2 (Text-Fig. 2).—In contrast to Experiment 1, indol was administered first to this animal while a normal diet was taken. As shown in Text-fig. 2 there was no increase in the excretion of bilirubin following this amount of indol, although the animal was observed for a control period of 33 days. No change in the levels of the blood or the reticulocytes took place. For the 15 days before the diet was changed 635 mg. of bilirubin were excreted, an average of 42 per day. The diet was then changed to that causing black tongue and a remarkably prompt and sustained increase in the excretion of bilirubin became evident. During the 15 days after the change 1,213 mg. of bilirubin were excreted, an average of 80 mg. daily or about double the levels of the control period. Concurrently there was a progressive drop in the blood values from 5,630,000 erythrocytes and 87 per cent hemoglobin to 4,100,000 erythrocytes and 70 per cent hemoglobin. At this point the bile became infected and the experiment was discontinued.

Experiment 3 (Text-Fig. 3).—The black tongue diet was fed for 14 days during which the excretion of bilirubin was extremely constant and the blood levels were stable. The total excretion was 753 mg. and the daily average 52 mg. Indol 250 mg. daily was then administered. The output of bilirubin rose promptly to a peak of 190 mg. daily and then stabilized at just over 100 mg., about double the output during the control period. The total output for the 14 days while indol was given was 1,701 mg. and the daily average 121 mg. The blood decreased from 4,370,000 erythrocytes and 67 per cent hemoglobin to 3,200,000 and 49 per cent hemoglobin. There was no significant change in the number of circulating reticulocytes. When the excretion of bilirubin and the blood levels had apparently stabilized, the amount of indol was increased to 500 mg. daily. This move resulted in an increase in the output of bilirubin to a total for 7 days of 1,406 mg., a daily average of 200 mg. At this point the blood had dropped to 2,700,000 erythrocytes and 39 per cent hemoglobin, again with no increase of reticulocytes. The diet was then supplemented with 10 gm. of liver extract daily, the administration of 500 mg. of indol daily being continued. Following the liver extract the reticulocytes rose on the 6th day to 9 per cent, and on the 8th day to 22 per cent followed by a progressive increase in blood levels. The output of bilirubin dropped progressively to an average of 100 mg. daily when infection intervened and the experiment was terminated. Here again the addition of liver extract reduced, by about 50 per cent, the hemolysis by indol.

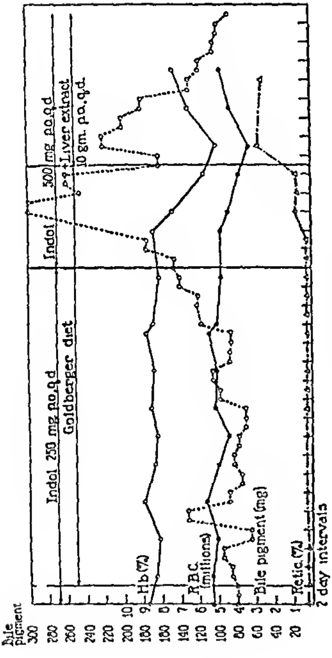
Experiment 4 (Text-Fig. 4).—In this experiment the administration of indol, 250 mg. daily, was begun while a normal diet was fed. During the control period without indol of 22 days, 1,643 mg. of bilirubin were excreted, a daily average of 74 mg. During a corresponding period of 22 days during which 250 mg. of indol were fed daily, the total output of bilirubin was 2,262 mg., a daily average of 101 mg. There was no apparent change in the blood. This animal showed a slight though distinct susceptibility to the hemolytic effect of indol even though on a normal diet. As the animal became adjusted to the presence of the toxin the



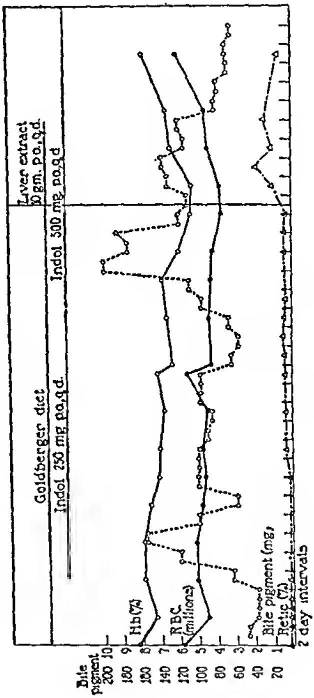
TEXT-FIG. 2. Dog 2



TEXT-FIG. 3. Dog 3



TEXT-FIG. 4. Dog 4



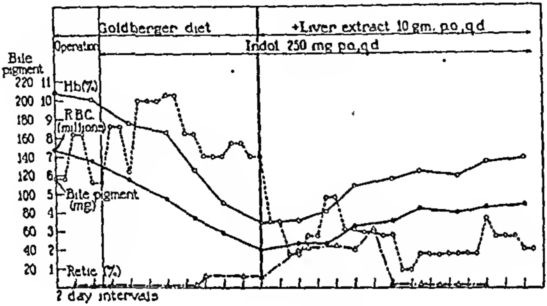
TEXT-FIG. 5. Dog 5

normal levels of the output of bilirubin were again restored, an average of 81 mg. a day for 20 days. At the beginning of Text-fig. 4, the diet was then changed to the deficient régime and the indol was continued. There ensued a slow but steady increase in the excretion of bilirubin over a period of 33 days. The total output for the first 10 days of this period was 868 mg. and for the last 10 days 1,160 mg. Coincidentally the blood dropped from 5,320,000 erythrocytes and 83 per cent hemoglobin to 4,800,000 and 81 per cent without any increase of reticulocytes. The daily dose of indol was then increased to 500 mg. The output of bilirubin for the next 10 days was 2,150 mg. and the blood dropped to 3,250,000 erythrocytes and 50 per cent hemoglobin. Without any other change 10 gm. of liver extract were administered daily. On the 2nd day the reticulocytes rose to 28 per cent followed by a rise in blood levels in 6 days to 4,200,000 erythrocytes and 65 per cent hemoglobin. The output of bilirubin dropped steadily to give a total of 1,657 mg. for the 10 days after liver extract, and to 608 mg. for the subsequent 6 days, an average of 101 mg. daily as compared to 215 daily before liver was given.

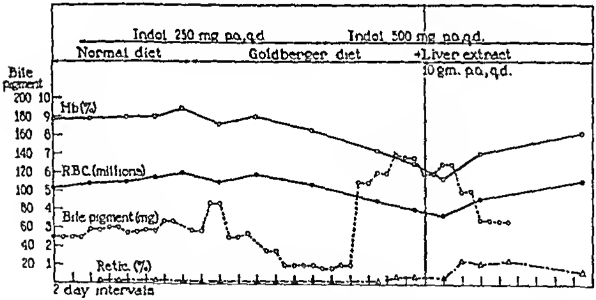
Experiment 5 (Text-Fig. 5).—This animal was fed the deficient diet for 1 month before indol was begun in a dosage of 250 mg. daily. During a control period of 12 days on this diet a total of 722 mg. of bilirubin were excreted, an average of 60 mg. daily. During the first 12 days of the indol feeding, 1,232 mg. of bilirubin were excreted, an average of 100 mg. daily. This increased hemolysis was compensated for by erythropoietic activity since no decrease of blood levels appeared, although the reticulocytes increased slightly in numbers. After 30 days the dose of indol was increased to 500 mg. daily. The output of bilirubin jumped to 1,706 mg. for the next 12 day period, an average of 140 mg. daily. The blood dropped from 4,390,000 erythrocytes and 67 per cent hemoglobin to 3,200,000 erythrocytes and 54 per cent hemoglobin without any increase in reticulocytes. Liver extract, 10 gm. daily, was then given. On the 2nd day following the reticulocytes were 9 per cent and on the 4th day 18 per cent. The output of bilirubin decreased somewhat more slowly than had been the case previously. For the first 10 days after the institution of liver therapy 1,208 mg. were excreted, a decrease of only 14 per cent, but in the next 7 days the output was 530 mg., an average of 75 mg. daily, a decrease of 50 per cent. Concurrently the blood rose in 6 days to 4,420,000 erythrocytes and 64 per cent hemoglobin and in the next 10 days to 5,400,000 erythrocytes and 75 per cent hemoglobin.

Experiment 6 (Text-Fig. 6).—This experiment is particularly instructive since the animal disliked the black tongue diet and took almost none of it after the biliary fistula was set up 1 month following the beginning of the diet feeding. Indol 250 mg. daily was started on the 6th postoperative day while the output of bilirubin was still in the 100 mg. range. The excretion of bile pigment rose sharply to a total for the next 17 days of 2,822 mg. or 166 mg. daily. The blood dropped from 6,700,000 erythrocytes and 101 per cent hemoglobin to 2,000,000 erythrocytes and 35 per cent hemoglobin. There was a slight rise of reticulocytes to 6 per cent. Liver extract, 10 gm. daily, was then administered, but the animal still refused to take the diet. The output of bilirubin dropped sharply neverthe-

less to a total of 646 mg. for the next 10 day period. On the 4th day the reticulocytes rose to 19 per cent, and on the 12th day to 30 per cent, then dropped quickly to normal levels. In the first 10 days the blood rose to 3,270,000 erythrocytes and 54 per cent hemoglobin, and in the next 10 days to 4,400,000 erythrocytes and 69 per cent hemoglobin.



TEXT-FIG. 6. Dog 6



TEXT-FIG. 7. Dog 7

Experiment 7 (*Text-Fig. 7*).—In this experiment after a primary control period of the normal diet 250 mg. indol daily were fed for 14 days. There was no increase in the excretion of bilirubin over the period without indol. The deficient diet was then fed and the small dose of indol continued. Deficiency developed so slowly however that no increase in the excretion of bilirubin was evident over a 15 day period. The indol was then increased to 500 mg. daily. During the previous

10 days the total output of bilirubin was 310 mg. During the next 10 day period the total output increased to 902 mg. or over 3 times, with a coincidental drop in blood levels from 5,490,000 erythrocytes and 82 per cent hemoglobin to 3,780,000 erythrocytes and 57 per cent hemoglobin, without any significant increase of reticulocytes. Liver extract was then given as a supplement to the diet in 10 gm. amounts daily without changing any other factor. The reticulocytes rose on the 5th day to 8 per cent and on the 7th day to 10 per cent. The output of bilirubin dropped steadily from the 4th day after liver was begun to an average of 78 mg. daily for the next 10 days. The blood rose to 4,640,000 erythrocytes and 70 per cent hemoglobin.

DISCUSSION

From the experiments the evidence is clear that an amount of indol which is well tolerated on a normal diet is causative of hemolysis and anemia when a deficient diet is fed. Furthermore the hemolytic as well as the anemia-producing effect of the combination of indol and a deficient diet may be prevented by supplementing the diet with liver extract. This is clear from the fact that following such supplement the output of bilirubin decreases to normal levels coincidently with the rise of reticulocytes and the improvement of the blood levels. It is to be inferred that the anemia results at least in part from hemolysis since that process is clearly conditioned by the dietary deficiency. The possibility exists, however, that a double process is operative, (*a*) the described increase of the hemolytic effect of indol on the deficient diet, and (*b*) a decrease in the erythropoietic power of the bone marrow. There is little evidence however that liver extract has the power to increase the rate of formation of erythrocytes since it is practically without effect in the standardized anemic dogs of Whipple (7). The anemia can be explained quite as well on the basis of simple destruction as by invoking two factors.

The mechanism of the increased hemolytic effect of indol in the presence of dietary deficiency is not clear. One possibility concerns the indol. (*a*) Under conditions of vitamin deficiency indol may be more freely absorbed than normally, (*b*) it may be metabolized to indoxyl more slowly than normally, or (*c*) an abnormal metabolism may be present by which some lytic intermediate product is formed. Experiments (8) have been made which show that no change in the rate of absorption or the conversion to indoxyl can be demonstrated even in a deficiency severe enough to cause black tongue. Moreover

it has not been possible to show any hemolytic effect of pure indol on washed erythrocytes *in vitro*. The possibility of the formation of pathological intermediate products is now under investigation.

A second possibility concerns a change in the erythrocyte itself as a result of the deficiency. It could become susceptible to lysis by indol although not normally so or it could be rendered by indol more susceptible to a normally existing bemolytic process. Both of these possibilities are now being studied.

An interesting phenomenon is the tendency of the organism to develop without treatment a slight but definite resistance to the hemolytic effect of indol. Reference to the figures shows that the increase in the rate of excretion of bile pigment is more marked immediately after the first administration of indol and then drops somewhat to a sustained effect. Tbis factor does not affect the validity of the conclusions however since sufficiently long periods were observed to rule out any spontaneous cbanges.

The changes in the reticulocytes in these experiments are also of interest. It is known from the work of Steele (9) and previous workers that bleeding or the hemolysis resulting from phenylhydrazine is attended by persistently elevated levels of reticulocytes. The factor of hemolysis in the experiments with indol is indisputable but essentially no elevation of reticulocyte numbers is encountered. One possible explanation is that the hemolysis by indol involves all the hemoglobin-containing cells including the reticulocytes, whereas bleeding or phenylhydrazine removes only adult cells, leaving younger forms in the circulation. Dock (10) states that doses of saponin may be administered which cause severe anemia with low numbers of reticulocytes. Were a reticulocytolytic action of indol operative the increase in numbers of those cells with the cessation of hemolysis following liver extract would be easily explained. They are being formed actually in greater numbers than normally but are not visible because they are destroyed as fast as formed. When lysis ceases, however, they appear for a brief period until the erythropoietic activity of the bone marrow slows down following the cessation of the constant drain upon it.

The explanation is suggested then that the anemia resulting from a deficiency of the vitamin and the administration of indol is a hemo-

lytic anemia, due possibly to an increased susceptibility to lysis of all hemoglobin-containing cells, including reticulocytes. The rise in blood levels following the administration of liver extract as well as the temporary increase of reticulocytes seems to reflect decreased hemolysis and a decreased, rather than an increased production of cells. No experiment made so far, however, has ruled out conclusively the possibility that a double factor is operative, (*a*) an increased rate of cell destruction and (*b*) a lessened rate of cell production, referable either to the toxic effect of indol or to the deficient diet. Further experiments bearing on this point are in progress.

CONCLUSIONS

1. Indol is more hemolytic in the presence of a deficiency complex than when a normal diet is fed.
2. The hemolytic effect can be abolished by supplementing the deficient diet with liver extract curative of pernicious anemia in man.
3. The hemolysis affects all hemoglobin-containing cells, including reticulocytes.
4. The repair of the anemia resulting from the administration of indol in the presence of a deficiency represents the cessation of a hemolytic process.
5. An abnormally low rate of production of erythrocytes may well be a factor in the production of the anemia.

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RESULTS OF THE INTRATRACHEAL INJECTION OF THE BORDET-GENGOU BACILLUS IN THE MONKEY AND RABBIT

By DOUGLAS H. SPRUNT, M.D., DONALD S. MARTIN, M.D., AND SARA McDEARMAN

(From the Departments of Pathology and Bacteriology, Duke University School of Medicine, Durham, North Carolina)

PLATES 10 AND 11

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Since the discovery of the Bordet-Gengou bacillus in 1906 (1), there have been a number of reports regarding the relationship of this organism to pertussis. The literature has been reviewed so extensively that only the recent work dealing with attempts to produce this disease in apes will be mentioned.

Sauer and Hambrecht (2) report that a paroxysmal cough developed in 5 cebus monkeys inoculated intralaryngeally and in 3 macaques after intranasal instillation. 2 of the macaques developed a lymphocytosis of over 20,000 cells per c. mm. The other macaque and the cebus monkeys with but one exception had a lymphocytosis of over 13,000. Culotta and his associates (3) were unable to obtain either lymphocytosis or cough in 12 *Macacus rhesus* monkeys which were inoculated intratracheally but were successful with 2 cebus monkeys. Sauer and Hambrecht (4) reported in detail the anatomical findings in a *rhesus* and a ringtail monkey, which were killed at the height of the experimentally induced disease and from which the Bordet-Gengou bacillus was recovered. Sections through the lungs of the *rhesus* monkey showed a number of pertussis organisms on the cilia and some of the bronchioles were blocked with mucus and cells. The alveoli and the interstitial tissue were in some areas filled with round cells, leucocytes, fibrin, erythrocytes, and pertussis-like organisms. Sections through the lungs of the ringtail monkey showed a similar but less marked picture.

Rich and his associates (5) by intratracheal injection of the Bordet-Gengou organisms were able to produce a lymphocytosis and a paroxysmal cough in chimpanzees but no anatomical studies were made.

From the work described above it is evident that the Bordet-Gengou bacillus can produce in monkeys a syndrome similar to that

seen in man. We have shown in a previous publication that this organism could produce an interstitial mononuclear pneumonia in the rabbit, and we thought it worth while to study in more detail the clinical and anatomical changes which can be produced in the monkey and also to extend our previous work with the rabbit (6).

Methods

Animals.—8 male and 3 female monkeys of the erythrocebus variety were used. The precise age of these animals could not be ascertained but from their behavior, weight, and white cell count we judged that we had 1 adult male monkey and 7 young ones. The female monkeys were apparently almost full grown.

Full grown rabbits weighing more than 2 kilos and shown by a method previously described (6) to be free from *Bacterium lepisepticum* and *Bacillus bronchisepticus* were used. The technique of inoculation and necropsy has been described in a previous paper (6).

Blood Counts.—The white blood counts were made just before the regular feeding time. The blood was drawn from an ear vein. In order to minimize the possibility of error the same pipettes were used for each monkey throughout the experiment. The differential formulae were computed from the study of 1,000 leucocytes. Peroxidase stains also were made and counted but these agreed in every instance with the results obtained with the Wilson stain.

As both the monkey and the rabbit are noted for the wide variations in their white cell counts, it was thought essential to determine the upper limit of the normal variation of these counts. Therefore, total and differential counts of 4 monkeys were made over a period of 10 weeks. Although the total counts were found to be exceedingly variable, none was found in which the lymphocytes exceeded 13,000 cells per c. mm. Scarborough (7), in his summary of the literature, found this to be true. We had available over 300 leucocyte counts and differential formulae for normal rabbits. The upper limit of the lymphocytes in these animals was 15,500 cells.

Diet.—The monkeys were kept in individual cages and fed once a day on a diet composed of bread, milk, bananas, and cod liver oil.

Cultures.—A number of strains of the Bordet-Gengou bacillus[1] were grown for 48 hours on the Bordet-Gengou medium to which 20 per cent human blood was added. The organisms were washed from the slants in saline and then injected intratracheally into rabbits. In Experiment 1 with monkeys 1, 2, 3, and 4 a strain was used which produced a good interstitial mononuclear pneumonia in rabbits. In Experiment 2 a mixture of 5 strains was employed. All of these strains both individually and when mixed produce an interstitial mononu-

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[1] These strains were obtained through the courtesy of Dr. W. A. Jamieson of Eli Lilly and Company.

clear pneumonia in rabbits. In Experiment 3, the organisms recovered from monkey 6 were used. The avirulent organisms which were used in certain of the monkeys and rabbits were obtained from Dr. J. A. Toomey. These were grown on the solid veal brain media described by him (8).

Inoculation.—The monkeys were lightly anesthetized with ether. The skin over the trachea was painted with tincture of iodine. A needle was then inserted through the skin into the trachea and the inoculum injected slowly. The animals were kept under the effect of ether for a few minutes to prevent their coughing up the material.

Necropsy.—Monkeys 1, 2, 3, and 4 were killed with chloroform and were necropsied at once. Monkeys 5, 6, 7, and 8 died and were necropsied at once, or within 3 hours after death. Since the use of chloroform in monkeys 1, 2, 3, and 4 might have resulted in the bacterial contamination of the lungs, the remaining monkeys, 9, 10, and 11, were killed by a sharp blow on the head. At necropsy the thorax was opened aseptically and a portion of the lung from the region of the lesion, if present, was taken for culture. This piece of lung was divided into 4 parts which were cultured aerobically and anaerobically in beef infusion broth with a pH of 7.4, on blood agar plates and on the Bordet-Gengou plates. In addition to these cultures, a long platinum loop was inserted deep into the bronchi and the recovered material streaked on blood agar and Bordet-Gengou media.

The lungs were inflated with air and fixed in Zenker's solution. Cross sections through the hilus were taken from each lung. After being embedded in paraffin, these were stained with hematoxylin and eosin, the MacCallum, and the Brown and Breen methods for bacteria, and for iron pigment.

Nasal Cultures.—Deep nasal swabs were cultured before the onset of the experiment for *Bacillus bronchisepticus* and *Bacterium lepisepticum* and none was found. After injection deep nasal swabs were taken daily. These were cultured on the Bordet-Gengou media.

EXPERIMENTAL

This experiment was designed to verify the observations of Sauer and Hambrecht (2) and Culotta and his associates (3), that the Bordet-Gengou organism would produce a pertussis-like syndrome in monkeys, and to study the anatomical changes in these animals. The clinical course and changes in the lungs will be outlined briefly.

Experiment 1.—4 monkeys were used in this experiment.

Monkey 1, weight 2,150 gm., was given 1 cc. of a suspension containing a 48 hour growth from 4 Bordet-Gengou slants. The lymphocytes in this animal never went above 12,000 cells per c. mm. This monkey was killed 14 days after inoculation with the Bordet-Gengou bacillus.

Necropsy.—Gross: The lungs were deeply pigmented and no mucoid material was found in the bronchi.

Microscopic: In many places the alveoli were filled with macrophages which contained hemosiderin. There was no marked increase in the number of cells in the interstitial tissue elsewhere.

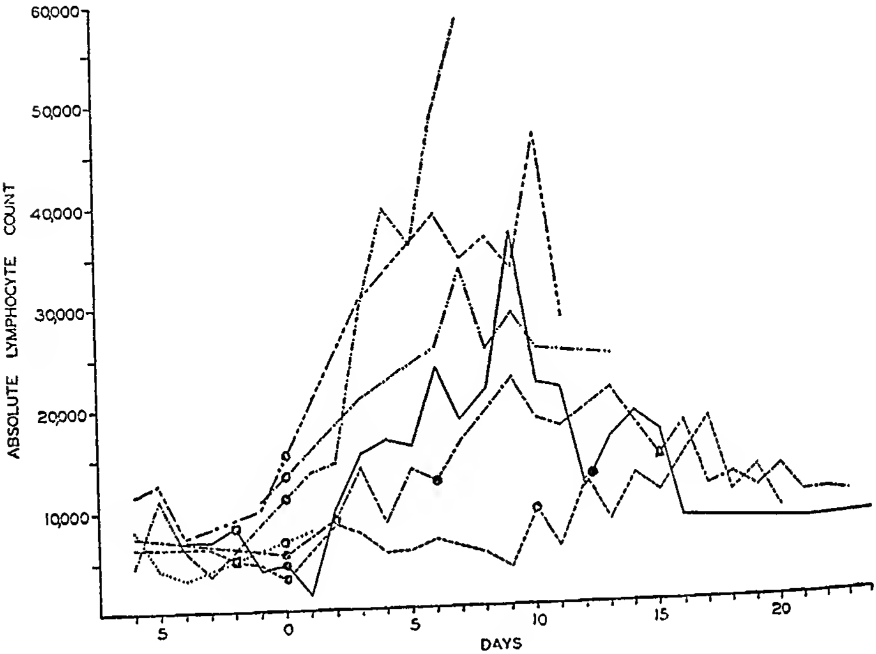

CHART 1

CHARTS 1 and 2. Monkey 2 Total leucocytes and lymphocytes
Monkey 3 Total leucocytes and lymphocytes
Monkey 5 Total leucocytes and lymphocytes
Monkey 6 Total leucocytes and lymphocytes
Monkey 9 Total leucocytes and lymphocytes
Monkey 10 Total leucocytes and lymphocytes
Monkey 11 Total leucocytes and lymphocytes

Virulent Bordet-Gengou bacilli injected ●
Avirulent Bordet-Gengou bacilli injected ▲
Staphylococcus toxin injected ■

Monkey 9 received one more injection of avirulent Bordet-Gengou bacilli which is not shown in the charts. This caused no change in either the leucocytes or lymphocytes.

Monkey 2, weight 900 gm., was given material from 2 Bordet-Gengou slants. The increase of lymphocytes was so slight that this animal was reinoculated with the material from 4 Bordet-Gengou slants. This resulted in a definite lymphocytosis, as shown in Chart 1. It was killed 14 days after the second inoculation.

Necropsy.—Gross: There was a small area of consolidation near the hilus. The lower lobes of both lungs were congested. The bronchi contained a tenacious yellow mucoid material.

Microscopic: In the lung there was an area, near the hilus, measuring about 10 x 4 mm. containing foci of necrosis. The tissue around these areas was filled

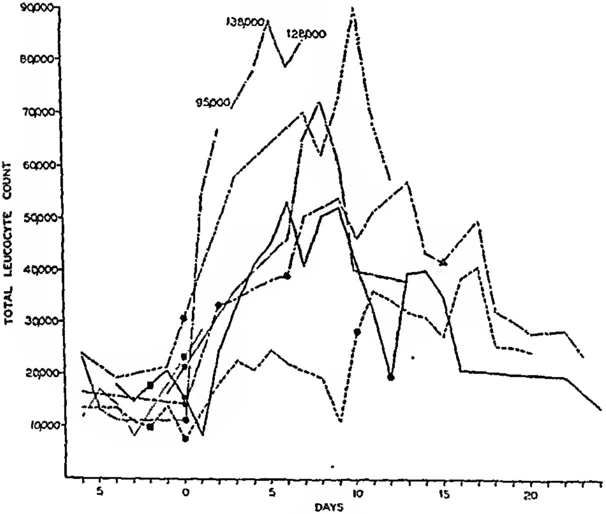

CHART 2

with mononuclear cells. The remainder of the lung tissue showed thickening of the interstitial tissue and some small focal accumulations of mononuclear cells. No bacteria were seen in the sections.

Monkeys 3 and 4, weight 1,300 and 1,350 gm., were both given material from 2 Bordet-Gengou slants and the results were essentially alike. Both monkeys were less active the day after injection and later were slightly sick. There was a definite lymphocytosis in both animals. The changes in monkey 3 are shown in Charts 1 and 2. The changes in monkey 4 were of the same magnitude but were omitted for the sake of brevity. They were killed 14 days after inoculation.

Necropsy.—Gross: The lungs were slightly congested and the bronchi contained a tenacious yellow mucoid material. Both lungs were easily inflated.

Microscopic: Throughout the lungs there was a definite increase in the number of mononuclear elements in the interstitial tissue. In addition to this there were focal accumulations of mononuclear cells, many of which were macrophages. They did not contain hemosiderin. The perivascular lymphatics also contained a number of cells and showed a slight increase of fibrous tissue around the bronchi. Figs. 3 and 4 show the characteristic picture seen in monkey 3.

Bacteriologic Report.—The Friedländer's bacillus, *B. coli* and *B. fecalis alkaligenes* were recovered from the lungs and bronchi of monkeys 1, 2, 3, and 4.

This experiment was done to determine the effect of larger amounts of the organism.

Experiment 2.—4 monkeys were to be used in this experiment. 2 of these died presumably of poison before the experiment was started but are included as controls.

Monkey 5, weight 1,000 gm., was given material from 8 Bordet-Gengou slants in 3 cc. of saline at 3 o'clock in the afternoon. The next morning it was inactive and would not eat. In the afternoon, it was lying down in the cage, vomited, and at times had a non-paroxysmal cough. At 10 o'clock the same night it was dehydrated from vomiting. 35 cc. of Locke's solution was given subcutaneously. The animal died a short time later (31 hours after injection) and was necropsied immediately. Charts 1 and 2 show the changes in the peripheral blood.

Necropsy.—Gross: At necropsy both pleural cavities were filled with cloudy fluid. The bronchi contained a mucoid material. The lungs were collapsed and red. Both lungs were easily inflated. A few round worms were found in the stomach.

Microscopic: A large proportion of the alveoli were filled with polymorphonuclear leucocytes and fibrin. The bronchi as a rule were free from exudate. A few, however, contained some fibrin. The remaining alveoli were filled with an amorphous granular material. A few swollen Gram-negative rods were found both in the alveoli and in the bronchi but cultures of this lung were sterile. The perivascular lymphatics contained a large amount of fluid but few cells.

Monkey 6, weight 1,100 gm., was inoculated with the same amount of material as monkey 5. The following day it was slightly lethargic but shortly regained its vitality. The only symptoms noted until the 6th day after inoculation were slight decrease in activity and loss of appetite. On the 6th day the animal began to vomit and had a slight wheeze and cough. Death occurred early on the morning of the 8th day after inoculation. The changes in the lymphocyte count are shown in Chart 1.

Necropsy.—Gross: The pleural cavities were filled with an opalescent fluid. The lungs could not be inflated and had firm lesions which extended from the hilus to the pleura. The bronchi contained a tenacious yellow mucoid material. Nothing more of interest was seen.

Microscopic: The alveoli throughout the sections contained a granular amorphous material and a few contained fibrin. A few small focal areas of necrosis were seen. The dominant feature, however, was the presence of large numbers of mononuclear cells, both in the alveoli and in the interstitial tissue. A few of these cells were lymphocytes. The majority of the cells, however, were either macrophages or monocytes. A few of the macrophages contained a small amount of hemosiderin. Red blood cells were seen in some alveoli, a few of which had been phagocytized. In addition to these elements there were some cells which were probably epithelial cells and some polymorphonuclear leucocytes. These were present in the interstitial tissue in the alveoli and in the bronchial epithelium. The perivascular lymphatics contained a number of mononuclear elements. No cells were seen in the bronchi but there was in places a considerable amount of amorphous debris. The changes in the lung are shown in Figs. 1 and 2. Embedded in the cilia of the smaller bronchi were found scattered small Gram-negative coccobacilli. An occasional Gram-positive coccus was seen. On the cilia of the larger bronchi there were areas which contained numerous Gram-negative bacilli, some of which were small coccobacilli. Most of the organisms were larger (2–3 μ and occasionally 4 μ by 0.8 to 1 μ) and stained irregularly, resembling the swollen degenerate forms seen in old cultures of bacteria. The streaked plate cultures showed innumerable colonies of the Bordet-Gengou bacilli and a few colonies of colon bacilli. Although it was impossible to determine whether or not these structures represented degenerate colon or Bordet-Gengou bacilli, the predominance of colonies of the latter on culture of this material added support to the view that these large bacterial forms were Bordet-Gengou bacilli.

Monkeys 7 and 8 are included as controls. After having been kept a month these animals began refusing their food. The next day they were quite ill and vomiting. Death occurred within 24 hours after the onset of the symptoms. These animals had been observed over a period of 8 weeks with blood counts twice weekly.

Necropsy.—Gross: The lungs inflated well and showed no changes. The liver was quite yellow. No other abnormalities were seen.

Microscopic: The alveolar walls were quite thin and no lining epithelial cells were seen. The bronchi and bronchioles were surrounded by a small amount of lymphoid tissue. The bronchial epithelium was well preserved and a moderate number of goblet cells were seen. The perivascular lymphatics were not dilated and contained no pus. The livers of both animals were almost completely necrotic. The convoluted tubules showed some necroses. It was thought that the animals were accidentally poisoned.

This experiment was designed to see if by damaging the lung we could not increase its susceptibility to the Bordet-Gengou bacillus. This was thought possible since pertussis is considered by some as being the result of a virus and the Bordet-Gengou bacillus. No virus

being available, it was decided to use staphylococcus toxin and multiple injections of the Bordet-Gengou bacilli as these had in earlier publications by us (6) been shown to cause anatomical changes similar to those caused by many viruses.

Experiment 3.—3 monkeys were used in this experiment.

Monkey 9, weight 1,800 gm., was given intratracheally 0.2 cc. of staphylococcus toxin. 2 days later the bacilli obtained from 4 Bordet-Gengou slants in 2 cc. of saline were injected intratracheally. This was repeated 12 days later and 17 days later the material from 6 slants of the avirulent organisms was injected. The animal was killed 11 days after the last injection. During the entire course of the experiment no changes were noticed in the monkey's behavior. The changes in the blood counts are shown in Charts 1 and 2.

Necropsy.—Gross: The lungs and other viscera showed no lesions.

Microscopic: The changes were essentially like those described for monkey 10.

Monkey 10, weight 1,800 gm., was inoculated intratracheally with the bacteria from 4 Bordet-Gengou slants in 2 cc. of saline. This same dose was repeated 2 and 4 days later. 9 days after this the material from 8 slants of the avirulent organism were given. The animal was killed 8 days after the last injection. The clinical course and the peripheral blood changes were similar to those of monkey 9, as is shown in Charts 1 and 2.

Necropsy.—Gross: The lungs and other viscera showed no changes.

Microscopic: There was some thickening of the interstitial tissue and a few focal areas where the alveoli were filled with mononuclear cells.

Bacteriological Report: The lungs and bronchi were sterile.

Monkey 11, weight 1,700 gm., was given 0.2 cc. of staphylococcus toxin. 2 days later the animal was given the bacteria from 12 Bordet-Gengou slants and 10 days after this the bacteria from 12 Bordet-Gengou slants. The monkey was killed on the 10th day after the last injection. There were no changes in the monkey's behavior. The blood changes are shown in Charts 1 and 2.

Necropsy.—Gross: No lesions were seen in either the lung or the other viscera.

Microscopic: The lung changes are essentially those seen in monkey 10.

Bacteriological Report: 25 colonies of the Bordet-Gengou bacilli were obtained. No other organisms grew out on the various culture media.

Summary of Results of the Experiments in the Monkey.—The intratracheal inoculation of cultures of Bordet-Gengou bacillus in the virulent phase caused a significant lymphocytosis in 6 out of 9 monkeys and an interstitial mononuclear pneumonia in 8 out of 9 instances. This pneumonia is microscopically composed of a large number of mononuclear cells which are in the alveoli and the interstitial tissue. These mononuclear cells are predominantly monocytes, macrophages,

and epithelial cells. There are, however, some lymphocytes and polymorphonuclear cells. When bacilli resembling the Bordet-Gengou bacilli are seen in the sections they are limited almost entirely to the cilia of the bronchi and bronchioles.

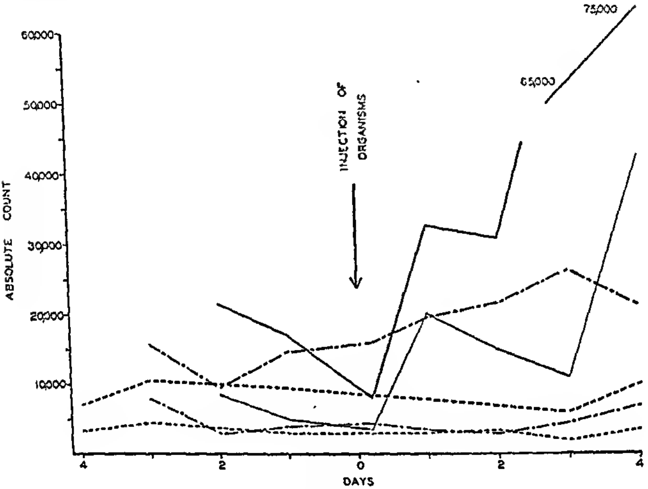

CHART 3

Rabbit receiving virulent Bordet-Gengou bacilli
Leucocytes ————
Lymphocytes ————
Rabbit receiving avirulent Bordet-Gengou bacilli
Leucocytes — — —
Lymphocytes — — — —
Rabbit receiving Friedländer's bacilli
Leucocytes - - - - - -
Lymphocytes ————

The following experiment was designed to see the effect of the Bordet-Gengou bacillus on the lymphocytes of rabbits. The animals all showed similar lesions to those described previously (6), therefore the morbid anatomical changes will not be described.

Experiment 4.—17 rabbits were inoculated intratracheally with virulent Bordet-Gengou bacilli. All of these animals showed a characteristic lymphocytosis, a typical example of which is shown in Chart 3.

This experiment was designed to show that the organisms other than the Bordet-Gengou bacillus found in the lungs at necropsy played no part in either the lymphocytosis or the interstitial mononuclear pneumonia.

Experiment 5.—6 rabbits were used in this experiment. 2 rabbits were injected with the material from 1 and 2 agar slants of the *B. coli*, 2 with *B. fecalis alkaligenes*, and 2 with Friedländer's bacillus. The lungs of all these rabbits showed extensive necrosis and polymorphonuclear reaction at necropsy and all a depression of the lymphocytes and an increase in the polymorphonuclear leucocytes as illustrated in Chart 3.

This experiment was designed to study the effect of the avirulent organism.

Experiment 6.—6 rabbits were given intratracheally various amounts of these organisms and 2 rabbits were inoculated with similar material heated for an hour at 60°C. The blood picture is shown in Chart 3.

Necropsy.—Gross: Most of the animals showed small lesions near the hilus. The microscopic preparations were similar to those seen when the more virulent cultures were used. The lesions, however, were less extensive. The characteristic lung picture is shown in Figs. 5 and 6. The heated organisms produced lesions similar in every respect to those produced by the unheated ones.

DISCUSSION

These experiments prove that the virulent phase of the Bordet-Gengou bacillus induces a lymphocytosis and an interstitial mononuclear pneumonia in the monkey and the rabbit. The lymphocytosis was shown to be significant; for, although both the monkey and the rabbit are subject to extensive variations in their white counts, these variations are much smaller than the increase produced by intratracheal injection of the Bordet-Gengou bacillus. The interstitial mononuclear pneumonia was present in varying degrees but always had the same characteristics. This pneumonia probably was the result of a toxic material liberated from the Bordet-Gengou bacillus. This is substantiated by the fact that in most instances no organisms were found and in the two instances in which the organisms were

present that they were limited to the cilia of the bronchi and bronchioles and were not found in the alveoli. The other organisms which were cultured from some of the monkeys were shown by experiment to play little if any part either in the morphological change or in the lymphocytosis and were probably only terminal contaminants. The possibility that the lung changes were the result of foreign material and not the toxin from the Bordet-Gengou bacillus was clearly ruled out in a previous publication (6). In these experiments, however, we found that under certain conditions living and heat killed cultures of avirulent Bordet-Gengou bacillus could produce a material, which by acting merely as a foreign body, caused a lesion in the lung similar to that produced by the toxic material of the virulent Bordet-Gengou bacillus. In this case, however, the lesion in the lung was not accompanied by a lymphocytosis and the organisms themselves were dead. This condition also was caused by Bordet-Gengou bacilli which had become completely avirulent. Toomey (8) has shown that these organisms can be recovered from the terminal stages of pertussis in man and that they have the ability to produce quantities of mucoid sticky material in culture.

That the Bordet-Gengou bacilli failed except in 2 instances to multiply in the lungs of the monkeys whereas they are found in large numbers in human pertussis was attributed to the higher susceptibility of man. This susceptibility might be explained by the theory that the spontaneous disease in man was the result of a virus-bacterium complex in which infection with the Bordet-Gengou bacillus was preceded by virus infection which sufficiently changed conditions in the lung to allow the bacteria to gain a foothold and multiply. Since we had shown (6) that staphylococcus toxin and the toxic material from the Bordet-Gengou bacillus could produce changes in the lung similar to those caused by many viruses, it was thought that we could damage the lung with these substances so that the Bordet-Gengou bacillus could gain a foothold. Hence several monkeys were given staphylococcus toxin and then a few days later the Bordet-Gengou bacillus. Other monkeys were given multiple injections of the Bordet-Gengou bacillus at intervals of several days. None of these procedures resulted in the organisms' multiplying in the lungs.

These experiments demonstrate the difficulty of producing pertussis in the monkey. This difficulty may be due to a higher resistance of the monkey to infection with the Bordet-Gengou bacillus or, in spite of our failure to obtain better results when the lung was damaged by toxins, to the possibility that a virus infection precedes the infection with the Bordet-Gengou bacillus.

Although we cannot say what the conditions are which make it possible for this organism to take hold, we believe that the following is a reasonable hypothesis for the development of the disease after the Bordet-Gengou bacillus gains a foothold and multiplies. In this multiplication it releases a toxic material which results in the lymphocytosis and the interstitial mononuclear pneumonia. At the height of the lymphocytosis some of the organisms, having become acclimatized to the host, become the avirulent form producing the sticky mucoid material referred to above. This material can also cause an increase in the amount of interstitial mononuclear pneumonia. This is essentially in agreement with the theory expressed by Toomey (8) in his study of the clinical course of the disease.

The cause of the paroxysmal cough has not yet been satisfactorily explained, but it seems likely since the Bordet-Gengou organisms apparently multiply best on the cilia of the bronchi and bronchioles that they must act as an irritant to cause some of the cough. The production of the mucoid material by the avirulent bacilli may also play a part in causing the cough. McCordock (9) has recently suggested that the cough is the result of damage to the sympathetic ganglion. We have not yet been able to find any confirmation of this possibility.

SUMMARY

Experiments are reported which show that the virulent Bordet-Gengou bacillus can produce a significant lymphocytosis and an interstitial mononuclear pneumonia in both the monkey and the rabbit. Both of these reactions occur apparently as the result of a toxic material formed *in vivo* from the Bordet-Gengou bacillus and are not dependent on the multiplication of the organism itself.

It was also shown that the strictly avirulent form could also cause an interstitial mononuclear pneumonia but no lymphocytosis. This

interstitial mononuclear pneumonia was thought to be the result of the foreign substance produced by the organism when it was in this stage. This was substantiated by the fact that this lesion could be produced both by living and dead organisms.

Since this paper was sent to press, Gallavan and Goodpasture (10) have called attention to a lesion in the epithelium of the bronchi and bronchioles which they have seen both in children dying of pertussis and in the chicken embryo infected with the Bordet-Gengou bacillus. This change consists of a necrosis and cellular infiltration of the epithelium of the bronchi and bronchioles. Further studies of the lungs reported in this paper show this lesion to be present at times in both the monkeys and the rabbits which received the virulent Bordet-Gengou bacilli. This lesion was particularly noted in monkey 6.

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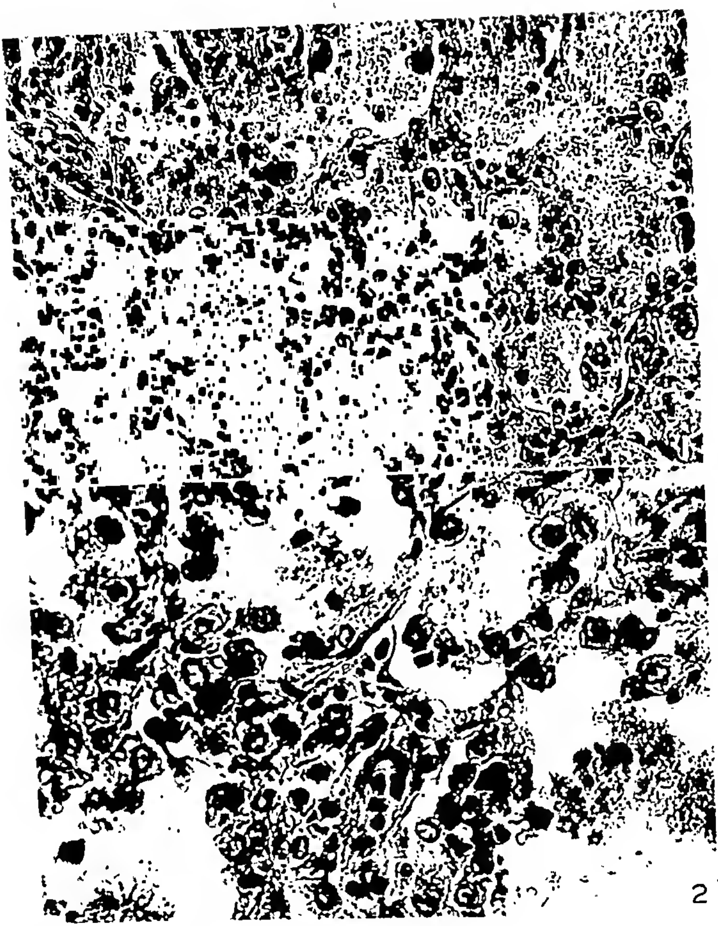
EXPLANATION OF PLATES

Plate 10

Fig. 1. Section from the lungs of monkey 6 which died 6 days after inoculation with virulent Bordet-Gengou bacilli, showing the characteristic interstitial mononuclear pneumonia. ×300.

Fig. 2. Same as Fig. 1, but showing better the mononuclear nature of the cells. ×660.



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(Sprunt *et al.*: Pertussis)

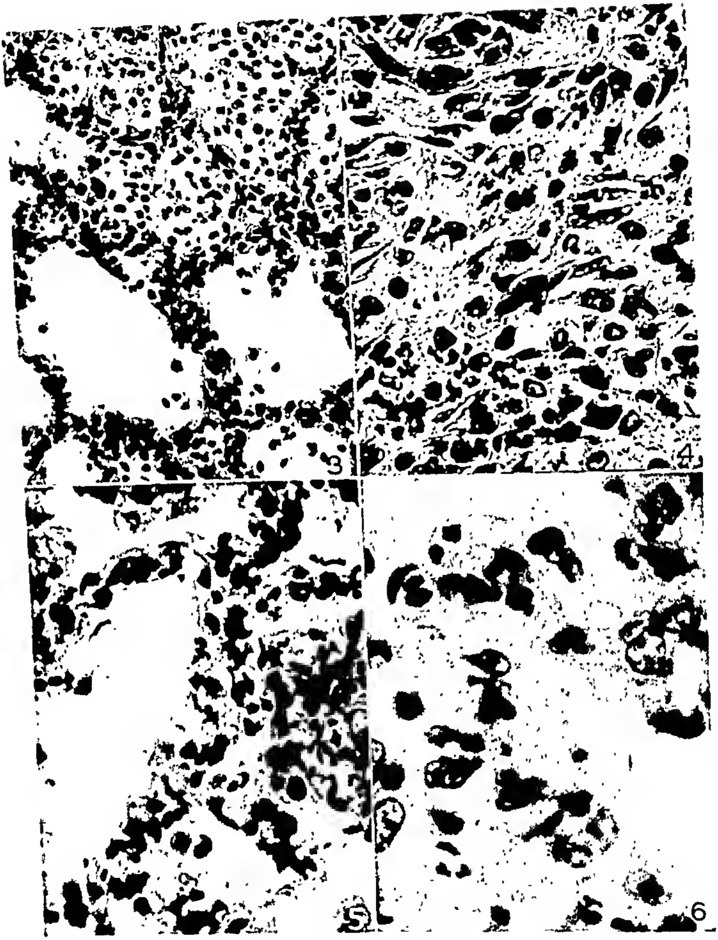
Plate 11

Fig. 3. Section from the lung of monkey 3 which was killed 14 days after inoculation with virulent Bordet-Gengou bacilli. Note here the dense accumulation of mononuclear cells. × 300.

Fig. 4. Same as Fig. 3 but showing cell types better. × 660.

Fig. 5. Section from rabbit's lung which had received avirulent Bordet-Gengou bacilli and was killed 5 days after injection. Note similarity of reaction to above pictures. × 300.

Fig. 6. Same as Fig. 5, but showing cell detail better. × 660.



(Sprunt *et al.*: Pertussis)

CANINE DISTEMPER IN THE RHESUS MONKEY (MACACA MULATTA)

By GILBERT DALLDORF, M.D., MARGARET DOUGLASS, AND H. E. ROBINSON, M.D.

(*From the Laboratories of Grasslands Hospital, Valhalla, New York*)

PLATE 12

(Received for publication, November 10, 1937)

Canine distemper has been described in various animals but never, to the best of our knowledge, in monkeys. Nicolle (1) inoculated one monkey with blood from a dog sick with distemper but the monkey remained well.[1] This appears to be the only effort to transmit the disease to monkeys, a surprising fact in view of the lively speculation that has at times centered about the nature of canine distemper.

Materials and Methods

Distemper virus was supplied to us in frozen dog spleen[2] and represents the strain or strains used in the manufacture of distemper vaccine. In most cases the virus was first passed to ferrets, using 0.5 cc. of the supernatant fluid of a 20 per cent emulsion injected subcutaneously into one or two animals and passed from ferret spleen to monkeys after the characteristic signs of distemper had appeared (reddening and swelling of the footpads, conjunctivitis, thick, mucoid secretion in the nostrils and coma). Ferret spleen was prepared in the same manner as the dog spleen.

20 per cent emulsions have regularly been used. The supernatant fluid has been given in doses of from 0.2 to 1 cc. For blood passage 4 to 7 cc. of freshly drawn blood has been given as a single dose. Inoculation has been successful by

[1] Baló, Josef, (Die unsichtbaren Krankheitserreger. Filtrierbare Vira, Berlin, S. Karger, 1935) quotes Veith as authority for the statement that the monkey is susceptible to distemper, but does not give the reference. Veith is not elsewhere quoted on the subject. The reference appears to refer to Joh. Elias Veith, author of Handbuch der Veterinärkunde issued last century. However no reference to the matter appears in either the edition of 1817 or 1842.

[2] Courtesy of Dr. Norman Pyle, Lederle Laboratories, Pearl River, N. Y.

TABLE I

Source of Virus and Route of Inoculation in 63 Successful Transmissions of Canine Distemper to Rhesus Monkeys

Source of virus	Route of inoculation					
	Intracutaneously	Subcutaneously	Intracerebrally	Intranasally	Subcutaneously and intracerebrally	Intraperitoneally
Ferret spleen		2	7	6	3	1
Dog spleen		6		4		
Monkey spleen		1		17		
Monkey blood		5				
Dried virus	2	4				2
Monkey brain			2			

Dried virus was desiccated dog spleen as prepared for the immunization of dogs against distemper.

TABLE II

Canine Distemper

Serial Transmission in Monkeys and Transmission from Monkey to Ferret

Pooled distemper dog spleen

↓

Monkey 1, inoculated subcutaneously with 0.5 cc. of the supernatant fluid of a 20 per cent emulsion of dog spleen. Developed fever after 5 days. Sacrificed on 6th day. Inclusion bodies present in tissues

↓

Ferret 1, inoculated with similar emulsion but prepared from spleen of monkey 1. Developed typical signs and febrile reaction of canine distemper. Sacrificed on 13th day. Inclusion bodies present in tissues

↓

Monkey 2, inoculated with supernatant fluid of a 10 per cent emulsion of ferret spleen 1 given intranasally by means of a ureteral catheter on each of 3 successive days. Developed typical signs and febrile reaction of distemper. Died on 15th day with massive tuberculous pneumonia. Inclusion bodies in tissues

Pooled distemper dog spleen

↓

Monkey 3, given 0.5 cc. of the supernatant fluid of a 20 per cent emulsion of dog spleen subcutaneously. Typical response Sacrificed on 12th day

↓

Monkey 4, inoculated similarly with an emulsion prepared from the spleen of monkey 3. This and two other animals similarly inoculated all developed usual fever and signs and recovered

↓

Monkey 5, inoculated subcutaneously with 4 cc. of venous blood taken from monkey 4, 7 days after inoculation. Distemper manifest in usual way. Sacrificed on 11th day. Inclusion bodies in various organs

↓

Monkey 6, one of eight animals in which three successive nasal instillations of a 10 per cent emulsion of monkey spleen No. 5 were given. All developed signs of distemper. No. 6 died on 20th day of the disease. Inclusion bodies numerous

all the routes tried. These, together with the sources of virus, are shown in Table I. Monkey tissues have frequently been tested for virus by inoculating ferrets. A typical example of transmission appears in Table II. Two failures to transmit the disease to ferrets have occurred, once when monkey spleen was used which was taken 12 days after inoculation and once 17 days after inoculation. All earlier transfers have been successful.

The nature of the disease in ferrets has been established by their clinical response and the presence of inclusion bodies in their tissues. These have regularly been found.

The only failures to transmit the disease by monkey tissue preparations occurred after brain and spleen had been stored for 167 days in 50 per cent glycerin. This experience is comparable to that of DeMonbreun (2) who found dog tissues lost their infectivity after 85 days storage under similar conditions.

An emulsion prepared from normal ferret spleen was given subcutaneously and intracerebrally into five monkeys. None developed a febrile response or symptoms of any kind.

Experimental Distemper in Monkeys

By these methods distemper has been transmitted to the 63 monkeys which form the basis of the present report. No appreciable difference in the response of the animals was associated with source of virus or method of inoculation. The group may therefore be described as a whole.

Clinical Course of the Disease

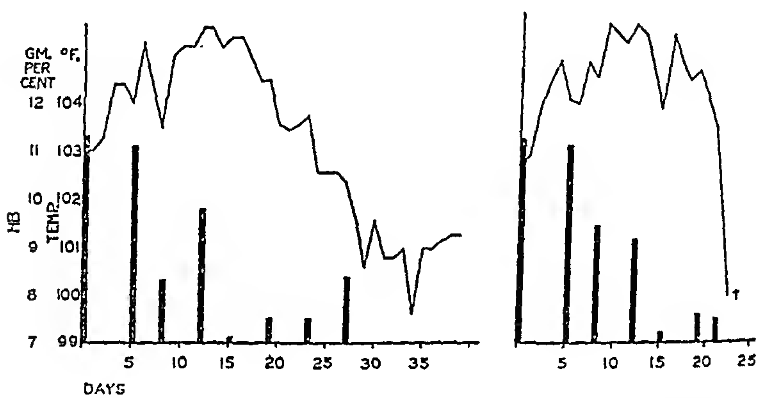
Based on our observations distemper in *rhesus* monkeys could be characterized as an acute, febrile disease having an incubation period of from 2 to 10 (average 5) days followed by 2 weeks of fever which in half the cases is distinctly biphasic and which is accompanied by extreme weakness, diarrhea, emaciation, hemoglobinemia and sometimes, in the early days of the febrile period rhinitis, conjunctivitis and irritability. The febrile period is usually followed by 1 to 3 weeks of subnormal temperatures. The mortality rate has been 9 per cent and the fatal outcome due to either encephalitis (three animals) or pneumonia (one animal).

The febrile response serves as an excellent guide to the course of the disease. Typical curves are shown in Text-fig. 1. The biphasic character of the first of those cases occurred in precisely half of our animals. The descent to normal or nearly normal may be of short duration, however, and our practice has been to measure the body temperature twice each day. This is all the more important

since the maximum temperature for any given day may occur in either the morning or afternoon.

Weakness appeared in one instance on the 5th day. Half the cases were first appreciably weak from the 9th to 11th days and the rest developed this sign during the following week. In most animals weakness became so marked that the monkeys hung limply from the walls of the runway when uncaged and often collapsed on the floor. In their cages they sat hunched and apathetic. Recovery, after the period of subnormal temperatures, usually arrived suddenly, and animals transferred to the open air pen quickly recovered their natural appearance and activity.

Loss of appetite and diarrhea occur during the period of weakness and even a robust animal rapidly wastes away until it resembles a terminal case of tubercu-



TEXT-FIG. 1. Changes in body temperature and blood hemoglobin in two monkeys suffering from canine distemper. The normal temperatures for these particular animals were 102.6° and 103°F.

losis. Loss of appetite is not as frequent as weakness. Often animals refuse to eat everything but fruit. The diarrhea is characterized by thin, yellow stools which the animal passes whenever disturbed.

Before these symptoms occur but by no means as commonly a degree of conjunctivitis may be present. Bright red streaks at the canthi of the eyes are often associated but these have frequently been seen in animals sick of other diseases. In one-fifth of our cases a few small vesicles were seen on the bridge of the nose or the lips and a thin watery secretion from the nares was about as common. Other symptoms which occurred were tremors, ataxia, cyanosis and irritability. Alopecia, described in distemper in dogs, also occurred but did not seem more severe than the confinement alone produced in control animals. The days on which these signs first appeared in a representative group of monkeys are shown in Table III.

TABLE III

The Days on Which Certain Symptoms Were Observed in a Group of 24 Monkeys Inoculated with the Virus of Canine Distemper

	Days																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Day fever occurred		5	3	5	1	5	2	1	2											
Duration of fever							1	1		1	1	3		5	1	3		3		5
Conjunctivitis				1	3		2	4		1			1	2						
Weakness									4	7		2	1	1		2				
Diarrhea								1	2	3		3	3		1					
Loss of appetite					3				4				1							
Nasal discharge			1		2	1	2		1											
Cyanosis					1	1										2	1			
Ataxia (?)									1	2						1				
Tremor									2											
Vesicles										1								1		
Irritability					1			1												
Paraocular swelling					1									1						

TABLE IV

Clinical-Pathological Observations in Three Cases of Canine Distemper in Monkeys

		Day of the disease									
		0	1	3	5	8	12	15	19	23	27
Blood sugar, *mg. per cent*	Case 1	83		84	89	74	83	77	78	94	94
	Case 2	83		74	85	65	100	91	89	84	105
	Case 3	83		89	91	63	105	88	95		
Hemoglobin, *gm. per cent*	Case 1	11.3		11.1	8.3	9.8	7.1	7.5	7.2	7.5	8.4
	Case 2	12.8			12.4	11.1	12.3	9.2	10.6	8.0	10.5
	Case 3	11.3			11.3	9.5	9.2	7.2	7.6	7.5	
Erythrocytes, *millions*	Case 1	5.0				4.7	4.4	4.8	5.0	2.1	3.7
	Case 2	4.4			4.8	4.6	4.9	4.9	5.8	5.2	5.3
	Case 3	4.9			4.0	3.9	4.3	5.1	5.2	3.7	
Leucocytes, *thousands*	Case 1	16.8		36	15.4	22.8	11.4	11.0	11.6	11.8	12.8
	Case 2	12.8	17	9.7	7.0	4.8	9.6	9.2	15.2	11	11.6
	Case 3	9.8	16.8	14.8	12.4	8.6	10.0	11.2	9.6	11.6	
Polynuclears, *per cent*	Case 1	55			45	21	21	29	30	40	29
	Case 2	51		58	12	47	43	43	41	42	37
	Case 3	56			57	40	51	60	56	47	
Lymphocytes, *per cent*	Case 1	41			58	72	77	71	66	55	66
	Case 2	44			42	83	51	55	55	56	55
	Case 3	39			37	49	48	40	37	44	

In view of the observations of Wharton and Wharton (3) that a constant manifestation of canine distemper in dogs is a reduction in erythrocytes, hemoglobin and blood sugar clinical-pathological studies were made in a number of animals. One group of three monkeys was periodically examined and the results of this study are incorporated in Table IV. In a group of twelve normal monkeys we found the average hemoglobin value to be 9.7 gm. per cent and the blood sugar 104 mg. per cent, values with which those in Table IV may be compared. The change in hemoglobin concentration in the peripheral blood has also been included in Text-fig. 1 to show the correlation between the anemia and the fever.

Cerebrospinal fluid pleocytosis has been observed in three monkeys in which repeated cisternal punctures were performed (Table V).

TABLE V

Increase in Number of Leucocytes in Cerebrospinal Fluid, in Distemper in Monkeys Following the Subcutaneous Inoculation of Ferret Spleen Emulsion

Animal	Normal	Number of days following inoculation		
		6	14	16
1	2	Bloody	30	36
2	4	6	10	56
3	7	22	1600	2110

Complications

Encephalitis was the cause of death in three of our animals, death occurring on the 18th, 23rd and 52nd days following inoculation. These animals were first stuporous and weak. They could be aroused only with difficulty, ceased to eat and slowly died after a period of falling temperature. Pneumonia occurred but once as a cause of death. The lungs were massively involved and showed characteristic lesions histologically. The animal had been dyspneic and cyanotic for several days. The same clinical features appeared in a few other animals which ultimately recovered.

Distribution of Virus

No deliberate effort has been made to study the distribution of distemper virus in the monkey. However a number of observations

have been made which indicate the periods in which blood, brain and spleen have been infectious, demonstrated either by passage to ferrets or to other monkeys in which the nature of the disease was later established by the presence of inclusion bodies. This information is summarized in Table VI.

Morbid Anatomy

Histological study of the tissues of animals sacrificed or dying on the 5th, 7th, 9th, 11th, 18th, 19th and 23rd days suggests a sequence of changes in the reticulo-endothelial tissues of considerable intensity. These may well be followed in the spleen. By the 5th day an intense congestion of the splenic sinuses is present which persists to the 19th day. The organ is engorged with blood cells and the sinus endothelium slowly thickens by proliferation about these masses of cells.

TABLE VI

Distribution of Distemper Virus in Infected Monkeys

	Days after inoculation tissues were demonstrated to contain virus										
	6	7	8	9	10	11	12	13	14	23	52
Blood	x	x			x	x					
Spleen						x	x				
Brain										x	x

By the 3rd week the endothelial reaction subsides and the pulp is marked by heavy deposits of hemosiderin.

The germinal centers of the follicles also respond by the 5th day. The earliest change seems to be necrosis of the germinal cells, slowly followed by an active regeneration and growth of the center. The total size of the entire follicle is not conspicuously enlarged, however, the increase in splenic size being due chiefly to the congestion.

Throughout the lymphatic tissue both in germinal centers of follicles and in the sinus endothelium, amorphous, neutral staining material is common which we believe represents necrotic endothelial cells.

The suprarenal cortex also shows changes related to the course of the disease. These consist of an early degeneration of the cortical epithelium, associated with the presence of inclusion bodies and soon

followed by intense subcapsular regeneration. The cells containing inclusions have commonly been in process of disintegration and careful examination is often necessary to determine whether an inclusion body is in the cytoplasm or the faded remains of a nucleus.

The changes in the central nervous system have been observed in so few animals it seems unwarranted to characterize them. The most conspicuous change we have seen has been enlargement and evidently multiplication of the larger glial elements. Perivascular cuffing and round cells in the substance of the brain have not been seen. The changes in the neurons have been limited to early stages of cell deterioration.

The most striking evidence of the disease is the occurrence of inclusion bodies. These have been identified in tissues from all animals in which death occurred before the 20th day following inoculation but not in cases dying later. They have also been present in tissues from ferrets, both animals infected with dog spleen and monkey tissues. In all cases they have been identical in appearance.

The inclusions are usually round or oval, occasionally shaped like an Indian club. They range in size from less than 1 micron to occupy the entire nucleus of an epithelial cell of kidney or suprarenal cortex. Usually homogeneous they sometimes have a darker center and are often surrounded by a halo. They stain brilliantly with acid dyes. They have been most numerous in the kidney and suprarenal and the germinal cells of the splenic follicles but have also been found in the lung, testis, brain and liver. They were numerous in the conjunctival margin of a ferret which had conjunctivitis. Most of the inclusions are intranuclear but in every case some have been seen in the cytoplasm and twice masses of them have been seen within kidney tubules. They are readily stained by Giemsa's mixture in Zenker fixed tissue but show to better advantage when stained by Laidlaw's method (4). In the central nervous system they are most numerous in Ammon's horn. A number of inclusion bodies are shown in Figs. 1 and 2.

The fatal case of pneumonia showed, in addition to the infiltration of the lungs, a serofibrinous pleurisy and pericarditis. The pneumonitis was distinctly interstitial in nature, a feature in harmony with reported observations in dogs.

Lack of Spontaneous Cases

A surprising feature of the present work has been the absence of cases of spontaneous infection. While simple precautions were taken

to prevent this, normal and infected animals were several times confined to the same room. Since all animals have had their temperatures measured twice daily and all were carefully watched throughout the experiments, it is very douhtful that cross infection occurred despite the intranasal administration of virus. This is in striking contrast to the behavior of ferrets and dogs.

DISCUSSION

The disease in monkeys seems distinctly more henign than in other reported hosts. The reported mortality in dogs is higher and in the ferret a fatal outcome is the rule. We have seen no seasonal fluctuation in virulence although the experiments have extended throughout a calendar year. At present we have no evidence that repeated passage in monkeys will increase the virulence of the infection, although the disease is being propagated with this in mind.

The infectiousness of the blood and the profound effects of the disease on the erythrocytes is interesting in light of the statement of Wharton and Wharton that distemper primarily affects these cells. The changes reported by these authors in the total leucocyte count and the lymphocytes closely correspond to our observations in monkeys.

It is not necessary to review at this time the similarity of the lesions and the appearance and distribution of the inclusion bodies to what occurs in dogs.

The evidence that the disease we have described is canine distemper may be summarized as follows:

1. It has regularly been produced by the inoculation of hacteriologically sterile suspensions of spleen taken from dogs sick with distemper.
2. It has likewise been regularly passed from ferrets suffering from distemper and showing the characteristic signs of that disease.
3. It has shown the characteristic biphasic fehrile response of distemper in half the cases.
4. It has heen passed to monkeys and back to ferrets with the development of distemper.
5. The lesions are undistinguishahle from those of distemper.
6. The disease has regularly heen associated, during the period of infectivity, with the presence of the inclusion hodies which occur in distemper.

7. The changes in the peripheral blood cells and blood sugar are identical with those described in canine distemper.

8. The cases with fatal issue have died of the fatal complications of canine distemper, encephalitis or pneumonia.

SUMMARY

Canine distemper has been transmitted to *rhesus* monkeys by a variety of methods. The disease is strikingly similar if not identical in its features with distemper in dogs.

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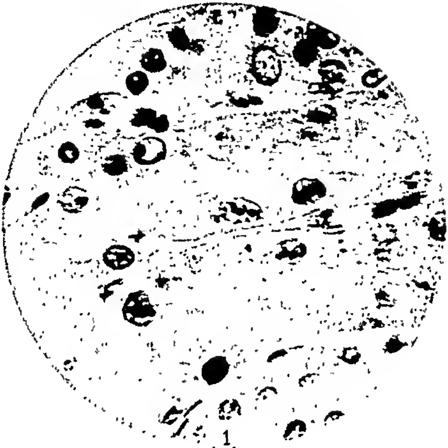
EXPLANATION OF PLATE 12

Typical inclusion bodies in monkeys inoculated with canine distemper.

FIG. 1. Inclusion bodies in kidney epithelium.

FIG. 2. Inclusion bodies in suprarenal cortex.



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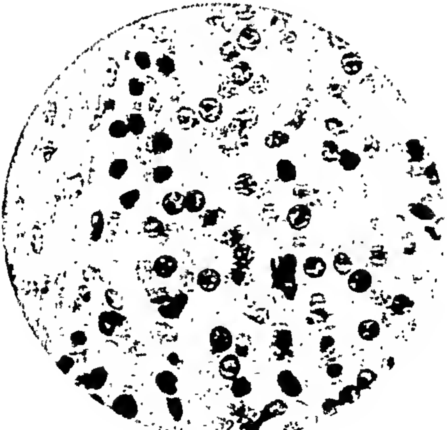

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(Dalldorf *et al.*: Canine distemper in *rhesus* monkey)

THE SPARING EFFECT OF CANINE DISTEMPER ON POLIOMYELITIS IN MACACA MULATTA

BY GILBERT DALLDORF, M.D., MARGARET DOUGLASS, AND H. E. ROBINSON, M.D.

(*From the Laboratories of Grasslands Hospital, Valhalla, New York*)

(Received for publication, November 10, 1937)

The present report is a detailed description of experiments in which monkeys (*Macaca mulatta*) suffering from canine distemper were found resistant to intracerebrally inoculated poliomyelitis virus, a phenomenon briefly reported previously (1).

Methods

Distemper was induced in monkeys by intracerebral (20 animals) or subcutaneous or subcutaneous and intraperitoneal inoculation of the supernatant fluid of a 20 per cent emulsion of ferret spleen taken from animals moribund with distemper. The preparation of the inoculum, the doses used and the responses in the animals were identical with the methods and observations described in the previous report (2).

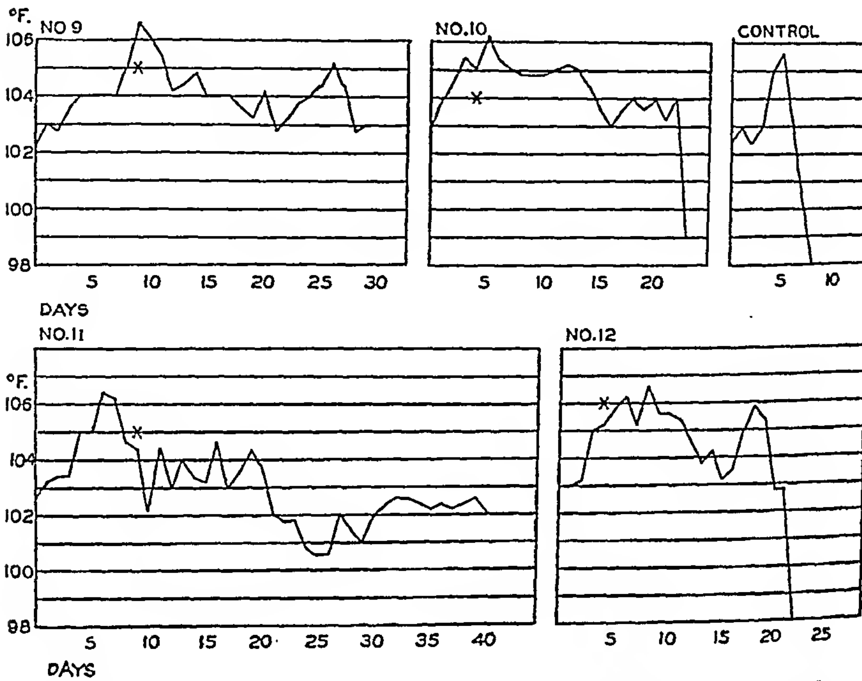
The poliomyelitis virus used in the present experiments was MV virus present in pooled, glycerinated monkey cord. Samples from various cords were taken, prepared in a 10 per cent emulsion which was centrifuged slowly for 10 minutes and given under light ether anesthesia into the cerebral tissue. The dose employed in the present experiments was 0.2 cc. or approximately 10 minimal lethal doses as based on our own titrations. When distemper was given intracerebrally the opposite hemisphere was used for the injection of poliomyelitis virus.

The virus of equine encephalomyelitis (eastern) was given as the supernatant fluid of a centrifuged emulsion (10 per cent) of monkey brain. The animals had been infected with guinea pig brain and both the monkeys and guinea pigs had behaved in typical fashion (3).[1]

The distemper vaccine used consisted of formolized dog spleen tissue. This was given subcutaneously over the flank. A second dose was injected after 14 days and this followed in another 2 weeks by an intradermal injection of splenic tissue dried in vacuum over phosphorous pentoxid and redissolved in saline before

[1] Virus supplied through the courtesy of Dr. Carl TenBroeck.

using. The distemper antiserum used was prepared from dogs immunized with distemper virus. It was given intraperitoneally in doses of 15 to 30 cc.[2]



Text-Fig. 1. The temperature records (degrees Fahrenheit) of four monkeys inoculated with distemper and, 9 days later, with poliomyelitis virus. The numbers will identify the animals in Table I. Nos. 9, 10 and 11 show the type of febrile response produced by distemper alone, No. 12 shows, 9 days after the inoculation of poliomyelitis virus, an abrupt rise in temperature which was accompanied by symptoms of poliomyelitis and led to death from poliomyelitis. Whether the rise in temperature in No. 9 on the 26th day is due to the same cause is unknown. No symptoms of poliomyelitis were recognized in this animal or Nos. 10 and 11. Death in No. 10 was due to pneumonia. The record of No. 11 has been continued to show the typical period of subnormal temperatures which occurs late in distemper in monkeys. The periods shown commence with the day on which the animals were inoculated with distemper. The day of inoculation with poliomyelitis virus is indicated by a cross.

The animals used weighed approximately 6 pounds and were maintained in clean and airy cages and carefully fed an excellent diet. Their temperatures were

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[2] All of these preparations were furnished through the courtesy of Lederle Laboratories, Pearl River, N. Y.

taken twice each day as routine, although the afternoon temperatures alone have been used in Text-fig. 1. The monkeys used in the present experiments were in excellent condition and the other element of critical importance, the poliomyelitis virus, was regularly and invariably producing a brief, fatal form of the disease at the time.

EXPERIMENTAL

Results of Intracerebral Inoculation of Poliomyelitis Virus in Monkeys Suffering from Distemper Produced by Intracerebral, Subcutaneous, or Subcutaneous and Intraperitoneal Inoculations of Distemper Ferret Spleen.—Six groups of monkeys inoculated with poliomyelitis virus at various intervals following inoculations of distemper virus, together with their controls, are represented by Table I.

As the table shows the concurrence of the two infections produced a greatly modified outcome of the poliomyelitis inoculation. Thus 12 of the 21 experimental animals recovered and half this number recovered without paralysis. The results also indicate that the most favorable interval is 9 days following the inoculation with distemper virus, a time when the lesions of distemper are at their peak of activity (2), when the fever has reached or just passed its maximum and when inclusion bodies are extremely numerous; in other words the period when the distemper is at a maximum of intensity. Of the four animals inoculated with poliomyelitis at this time two recovered without evidence of poliomyelitis, a third died of an extensive lobar pneumonia, having shown no evidence of poliomyelitis, and one died of the latter disease. Study of the temperature records in these cases clearly indicates the maximal sparing effect. Thus animals which die of poliomyelitis or develop extensive paralyses have always shown an abrupt rise in temperature after a suitable period of incubation, which may easily be identified even when superimposed on the natural febrile reaction of distemper. The records of the four animals in this group have been redrawn (Text-fig. 1) to illustrate this point.

As is also evident from Table I, but even more so from the individual records the presence of distemper greatly modified the course of poliomyelitis even when death was not prevented. Thus death was delayed on an average 8 days among the experimental animals and the febrile response to poliomyelitis usually modified in that the sud-

TABLE I

Effect of Inoculation of the Virus of Poliomyelitis in Monkeys Ill with Canine Distemper and the Resistance of the Survivors to Reinoculation

Animal	Time after distemper inoculation that poliomyelitis was given	Duration of incubation of poliomyelitis before paralysis	Extent of paralysis	Outcome		Results of reinoculation	
				Recovered	Died	Resistant	Susceptible
	days	days			days		
1	4	No paralysis	0	x			x
2	4	13	+	x			
3	4	13	++++		13		
4	4	13	++	x			
Control		7	++++		10		
5	7	13	++	x		x	
6	7	13	+++	x		x	
7	7	No paralysis	0	x			
8	7	12	++++		24		
Control		8	++++		20		
9	9	No paralysis	0	x		x*	
10	9	" "	0		16†		
11	9	" "	0	x			x
12	9	12	++++		21		
Control		8	++++		11		
13	13	15	++	x		x	
14	13	No paralysis	0	x			x
15	13	7	++++		9		
16	13	No paralysis	0	x			x
Control		7	++++		10		
17	20	11	++++		20		
18	20	12	++	x		x	
19	70-20	13	++++		32		
20	70-20	8	++++		16		
21	70-20	9	++++		32		
Control		8	++++		13		

* Recovered with paralysis.

† Death due to lobar pneumonia.

den drop which presages death in control animals was delayed and less sudden.

The results also indicate that the sparing effect had largely subsided

by the 20th day, that is at the end of the febrile period of distemper.

What the table does not show, but which the records in Text-fig. 1 are representative of, is that while the distemper modifies the poliomyelitis the reverse is not true, the duration and degree of fever, the subsequent period of subnormal temperature are not shortened or modified in the least by the poliomyelitis.

Course of Experimental Poliomyelitis in Monkeys Convalescent from Canine Distemper, Having Had Distemper Vaccine or Receiving Distemper Antiserum.—The course of poliomyelitis produced by intracerebral inoculation of MV virus in monkeys convalescent from distemper is shown in Table II. In it are included the salient features of poliomyelitis occurring in monkeys which had had distemper at various times, monkeys which had distemper vaccine and attenuated virus and four animals which received distemper antiserum.

The results indicate no significant resistance to poliomyelitis as a result of these various experiences with distemper or as a result of antiserum unless the two animals which had complete courses of distemper vaccination do indeed indicate an altered resistance. Unfortunately the group was small. That both animals behaved in almost identical fashion and that their controls developed typical poliomyelitis justifies some consideration.

The only other group which behaved in atypical fashion comprised three monkeys which had distemper as a result of intracerebral inoculation, which 53 days later were reinoculated with distemper and which 20 days later were inoculated with poliomyelitis. The modified course of the poliomyelitis in these animals could well be due to the second distemper episode, since partial protection may be observed in Table I in animals inoculated 20 days following distemper.

The ineffectiveness of distemper antiserum is quite evident from Table II. Serum from distemper convalescent monkeys has likewise been found to have no neutralizing properties when mixed with equal parts of a suspension of poliomyelitis virus and stored overnight.

Results of Reinoculation with Poliomyelitis in Monkeys Convalescent from Concurrent Distemper and Poliomyelitis.—Nine of the survivors of the experiments included in Table I were subsequently reinoculated with poliomyelitis by the same route and with a similar preparation of virus as that first used. Four of the animals showed no febrile

or symptomatic response to this inoculation, four developed typical attacks of poliomyelitis and succumbed. One animal developed a febrile response but recovered with paralysis. The resistant animals

TABLE II

The Course of Experimental Poliomyelitis in Monkeys Which Had Recovered from Distemper, Had Been Inoculated with Distemper Vaccine or Were Treated with Distemper Antiserum

Animal	Previous experience	Duration	Comment
		days	
1	Antiserum at time of inoculation	7	Typical
2	" " " " "	9	"
3	Antiserum during preparalytic stage	8	"
4	" " " "	8	"
Control		8	"
5	Complete course of vaccine with febrile response. Poliomyelitis 1 mo. later	30	Atypical
6	" "	30	"
Control		9	Typical
"		5	"
7	Dried virus. Brief febrile response. Poliomyelitis 1 mo. later	12	"
8	" "	6	"
9	" "	13	"
Control		13	
"		5	"
"		7	"
10	Distemper. 10 wks. later poliomyelitis	11	"
11	" " " " "	11	"
12	Distemper. 4 mos. later poliomyelitis	6	"
13	" 5 " " "	13	"
14	" " " " "	12	"
Control		7	"

Vaccine and antiserum were furnished by Lederle Laboratories.

were all cases which had been paralyzed by the first experience with poliomyelitis, the susceptible animals were in each case animals which had been completely spared, in which the temperature records gave no clue to a response to poliomyelitis and in which no paralysis was

observed. The exception was an animal which recovered from the primary attack without paralysis but in whose records it was at one time noted that some weakness was present in a leg. This disappeared shortly afterward and no residual paralysis was observed. These records are likewise incorporated in Table I.

Course of Experimental Poliomyelitis in Monkeys Suffering from Vaccinia Encephalitis and the Concurrence of Poliomyelitis and Equine Encephalomyelitis.—Six monkeys inoculated intracerebrally with MV virus were subsequently inoculated with 0.2 cc. of the supernatant fluid of a 10 per cent emulsion of monkey brain taken from a fatal case of equine encephalomyelitis. Three animals received the second disease 2 days after the poliomyelitis inoculation and the other three after 3 days. Death occurred in the first group 6, 6 and 7 days following the original inoculation or 4, 4 and 5 days after the inoculation with encephalomyelitis. In the second group death occurred 3, 5 and 6 days after the encephalomyelitis inoculations. Two control cases of poliomyelitis died on the 6th and 13th days and two control cases of equine encephalomyelitis on the 4th day following inoculation. It was evident from the foregoing that the poliomyelitis did not curb the course of the encephalomyelitis and that the latter did not modify the development of the poliomyelitis in a significant degree.

Two animals suffering from vaccinia encephalitis were likewise inoculated with poliomyelitis virus and promptly succumbed with poliomyelitis, indicating that the former did not exclude the development of the latter.

The Results of Injection of Normal Ferret Spleen.—As a further control of the present studies 0.2 cc. of the 20 per cent supernatant fluid of a centrifuged, 20 per cent emulsion of normal ferret spleen was injected intracerebrally into each of five monkeys. In no case did a febrile response develop and in each instance (three animals) in which poliomyelitis was inoculated after an interval of 7 days typical and fatal quadriplegia developed.

DISCUSSION

The evidence presented indicates that during the course of distemper in the monkey, that animal is highly resistant to the develop-

ment of experimental poliomyelitis, that the resistance reaches a maximum during the 2nd week of the distemper and that the characteristic feature of the resistance is that many of the animals are spared an experience with poliomyelitis intimate enough or massive enough to produce the typical disease, to produce any clinical manifestations at all, or to lead to a fixed immunity to poliomyelitis. With this interpretation in mind it has seemed wise to speak of the phenomenon as a sparing effect until the precise mechanism is better understood.

The protective effect of distemper is intimately associated with the disease itself and disappears during convalescence. The response of distemper immune monkeys to poliomyelitis is not known since to date no solid, fixed immunity to the disease has been observed in our animals. However, it is possible that an immunity would influence the course of poliomyelitis, since two animals which had had protracted contact with attenuated distemper virus appeared to show some resistance to poliomyelitis similar to the effects of pseudorabies in animals immune to B virus (4).

Since the original observation (1) that such a sparing effect did exist between these two diseases, Findlay and MacCallum have reported a similar phenomenon (5). They observed that if Rift Valley fever virus were injected intraperitoneally in monkeys and followed in 2 hours by inoculation of pantropic yellow fever virus, approximately 60 per cent of the animals survived the yellow fever. This was an extension of the work of Hoskins (6) who reported the year previous that a neurotropic strain of yellow fever virus protected 60 per cent of monkeys from the pantropic virus. In this case the interval had to be less than 20 hours to be effective. Judging by the protocols of these experiments the phenomenon would seem to be like that we have observed. Also of possible interest to the discussion are the studies of Magrassi (7) and Doerr and Seidenberg (8) on the effects of double inoculations of rabbits with encephalitic strains of herpes virus. Magrassi observed that if such a virus be injected peripherally and followed on the 7th or 8th day by an intracerebral inoculation of the same virus the two nullified each other, whereas each was capable of producing fatal encephalitis if given alone. This was confirmed by Doerr and Seidenberg who also showed that the first inoculation might be intracorneal and produce the same result.

Possibly related studies are those of Collier (9) who observed an increased rate of disappearance of fowl plague virus in rats which had, 14 to 54 days previously, been injected with fowl plague, rabbit myxoma or Rous sarcoma virus. The evidence in these experiments is not extensive enough to justify a close comparison.

In discussing their results Findlay and MacCallum point out that it has been repeatedly observed that certain closely related virus diseases of plants, yellow mosaic and tobacco mosaic, two different strains of X virus of potatoes, etc., are mutually exclusive, that the presence of one prevents the growth of the other.

The consensus of opinion appears to be that in the case of the plant viruses the strains must be generically related to produce this effect. This relationship was of course present in Hoskins' work in which strains of yellow fever virus were employed. As Findlay and MacCallum point out there are many points of similarity between Rift Valley and yellow fever, clinical symptoms and morbid anatomy being quite similar, the same species of monkeys being susceptible to both, both being transmitted by aedes mosquitoes and both having neurotropic strains. However no cross immunity has been demonstrable by serological tests, or *in vivo* immunity in man or other tested animals, and the pathogenic range is quite dissimilar. Findlay and MacCallum found that inactivated neurotropic virus, normal brain tissue and fowl pest virus were ineffectual.

Closely related diseases or strains of the same disease have therefore been the rule in the experiments in which a sparing effect has been noted. This point is of interest in the light of the present report, suggesting, as it does, a relationship between poliomyelitis and canine distemper. Such has indeed been suggested on several occasions but no significant evidence has been collected to support the view. Of our own observations there is little to indicate a similarity. The clinical responses and sequelae are different, as are the distribution of virus and presumably the lesions of the central nervous system although the evidence on this point is still too limited to be of much weight. No cross immunity has been demonstrated to date. Indeed the sole point of similarity which we have observed has been the reaction of the reticulo-endothelial structures which seems quite similar in both diseases.

The possibility that the effect is due to temperature alone seems precluded by the reported failure of hyperpyrexia (10) as a treatment of experimental poliomyelitis as well as isolated cases in which animals recovered during a period when the febrile reaction was insignificant.

Findlay and MacCallum suggested that the explanation of their experiments was a cellular blockade against the second virus. Reticulo-endothelial blockade with India ink proved ineffective. Reticulo-endothelial blockade may well exist in animals suffering from distemper: the changes in the splenic sinuses are very suggestive.

Another possibility is that both viruses require, for their propagation, a common cell protein or other substance which the conjugation of the first virus exhausts and thereby prevents the multiplication of poliomyelitis virus.

The designation of this phenomenon as a sparing effect seems justified by the observation that recovered animals have evidently been spared an intimate contact with poliomyelitis virus, and hence are still susceptible to it, and because of the desirability of clearly distinguishing this immunity mechanism from those due to the development of tissue resistance or serological immune substances, neither of which occur in the present instance.

CONCLUSIONS

1. *Rhesus* monkeys inoculated with canine distemper are relatively or completely immune to experimental poliomyelitis during the first 2 weeks of the distemper.

2. Monkeys convalescent from distemper are not resistant to experimental poliomyelitis.

3. Two monkeys vaccinated with distemper virus responded to poliomyelitis in a modified manner.

4. Distemper antiserum did not influence the course of experimental poliomyelitis in *rhesus* monkeys.

5. Equine encephalomyelitis and vaccinia encephalitis showed no sparing effect on the course of experimental poliomyelitis.

6. The concurrence of distemper and poliomyelitis in monkeys seems to represent a new immunity mechanism in the virus field.

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BLOOD PLASMA PROTEINS AS INFLUENCED BY INTRAVENOUS INJECTION OF GUM ACACIA

By G. P. HECKEL, M.D., C. C. ERICKSON, M.D., C. L. YUILE, M.D., AND R. E. KNUTTI, M.D.

(From the Department of Pathology, The University of Rochester School of Medicine and Dentistry, Rochester, New York)

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Any experimental technique which involves disturbance of liver function is of particular interest in regard to plasma protein studies, for there is evidence that the liver is associated in the production of plasma protein. When a solution of gum acacia is injected intravenously in the dog, there is a marked decrease in the blood plasma protein concentration (5). That the liver is concerned with this process is suggested by the observation that injected gum acacia is rapidly removed from the blood and deposited to a large extent in the liver, whence it is slowly eliminated in the bile (4).

In any attempt to evaluate this phenomenon following gum injection, it is important to ascertain, first, whether decrease in plasma protein *concentration* is associated with a decrease in *total* circulating protein, and second, the degree of impairment of liver function under these conditions. The data given here represent such an attempt.[1] By means of plasma volume determinations it was possible to show a decrease in the total circulating protein. Determinations of plasma fibrinogen were made as a means of estimating the relative degree of hepatic function. Marked disturbance in fibrinogen concentration was demonstrated both in dogs receiving single injections of the gum, and in those in which repeated doses were given. It was possible by means of repeated weekly injections of acacia to maintain dogs at low plasma protein and fibrinogen levels for several weeks. Following

[1] We are grateful to Eli Lilly and Company for furnishing valuable material used in these experiments.

such procedure, the most marked anatomical changes were noted in the liver.

Methods

In experiments involving a single injection of gum acacia four normal dogs were used. These animals were given a standard potato-hamburger diet containing all food elements essential for health. The diet which was adjusted to furnish 2 gm. of protein per kilo of body weight, was usually started a few days before the proposed injection. Basal figures as indicated in the tables were obtained by one or more analyses previous to injection. When more than one analysis was made, the average is given. The dogs were put in metabolism cages. In two of the animals analysis of urine for nitrogen was done. The urine was collected in concentrated sulfuric acid, and nitrogen was determined by the micro-Kjeldahl method, to be described.

Blood for analysis was collected in 1.4 per cent sodium oxalate solution in hematocrit tubes. Appropriate factors for dilution were introduced into the various formulae.

Plasma volume determinations were made by two methods. In two of the dogs (36-21, 36-94), the method of Hooper, Smith, Belt, and Whipple (7) with modifications by Whipple and Robscheit-Robbins (13) was used. In dog 35-151 we employed a modification of Gibson and Evans' method (6) using T-1824 dye, and spectrophotometric quantitation. Nitrogen determinations were made in duplicate and triplicate by a modification of the method of Goebel, as described by Peters and Van Slyke (10).

Albumin and globulin were determined by Howe's method, as described by Peters and Van Slyke (10), using 22 per cent sodium sulfate at 37°C. Fibrinogen was estimated by the following method: To 1 ml. of oxalated plasma in a large test tube, 28 cc. of 0.8 per cent sodium chloride solution is added. After mixing, 1 ml. of 2.5 per cent calcium chloride solution is added, mixed, and allowed to stand for 1 hour. The resultant clot is washed several times and transferred to a micro-Kjeldahl flask, where nitrogen is determined by the usual method. Duplicate samples were determined.

Preparation of Acacia Solutions.—A sterile solution of gum acacia (Lilly—"without sodium chloride") of 30 per cent concentration is diluted with Locke's solution (minus calcium chloride) to yield the desired amount of acacia in the concentration desired. One dog (32-8) was given 6 per cent solution, while all other animals received the substance in 12 per cent concentration. The solution is heated to body temperature and administered slowly into the jugular vein.

To determine if the presence of acacia might have any possible interference with the precipitation of globulin, or with the precipitation or clotting of fibrin *in vitro*, tests were carried out using a mixture of the gum solution in various concentrations with plasma of determined fibrinogen and albumin content. Differences were negligible and entirely within the limits of experimental error for the various techniques.

EXPERIMENTAL OBSERVATIONS

Dogs Receiving Single Injections of Gum Acacia.—Four animals were studied in this group. All dogs were on the same diet, which with one exception (35-151) was continued throughout the course of the experiment. All dogs received the same relative amount of 12 per cent gum acacia solution (1 gm. of acacia per pound body weight).

TABLE 1

Single Injection of Gum Acacia

(A) Dog 36-21.

Day	Plasma protein		Blood plasma volume	Red blood cell volume	Total blood volume	Hematocrit plasma	Urine (48 hrs.)	
	Concentration	Total in circulation					Volume	Nitrogen
	gm. per cent	gm.	cc.	cc.	cc.	per cent	cc.	gm.
Basal	5.95	32.3	544	643	1187	43.1		
3	4.26	23.3	547	545	1092	50.4		
5	4.90					45.7		
6	4.84	31.9	661	535	1196	45.1		
8	4.73	25.2	533	511	1044	51.0		
10	6.05	26.7	422	476	918	48.1		
(B) Dog 36-94.								
Basal	6.60	54.4	828	1061	1889	44.3	1650	12.8
1	4.73	43.6	923	926	1849	49.9		
2	4.80					51.2	1400	12.4
3	4.53	48.0	1059	1051	2110	51.7		
4	4.76					50.8	1750	12.2
5	4.97	51.8	1138	1201	2339	48.6		
6	5.10					50.6	1400	13.5
7	5.06					47.0		
8	5.38					48.0		
10	5.27	45.6	865	865	1730	50.0		
14	5.86	47.8	816	729	1545	52.8		

Table 1 (A) shows the reaction of dog 36-21 to the injection. Daily determinations were not done on this animal, the interest at the time being in blood volume and total protein changes at time intervals that had previously been shown to correspond to maximum and minimum levels. It will be noted that there was practically no in-

crease in the plasma volume on the 3rd day following injection, at which time the plasma protein concentration was at the lowest point. On the 6th day, however, there was an appreciable increase in plasma volume, the plasma protein concentration having risen in the meantime. On the 10th day, when the plasma protein concentration was up to the normal range, the plasma volume and blood volume were below their original levels. This ultimate shrinkage of blood volume is a phenomenon which has been observed to a greater or less extent in all three of the dogs on which blood volume determinations have been made. We attempt to explain it on the basis of diminution of total colloid, both plasma protein and acacia. That there is a diminution of total plasma protein, is indicated by the results. Acacia at this time should (3) be largely gone from the blood stream. In this event, then, the lowered amount of colloid would carry with it less fluid, thus accounting for the lowered plasma volume.

An interesting observation brought out by this and other tables has to do with the relationship between the hematocrit plasma percentage and the estimated plasma volume. It is to be noted that changes in plasma percentage were not proportional to changes of the plasma volume. For example in Table 1, from the 6th to the 8th days, the plasma per cent increased from 45 to 50 per cent, while the plasma volume diminished from 661 to 533 cc. There was also a slight decrease in total blood volume and in the red cell volume. The significance of this change is not understood but it has been so constant that it cannot be overlooked. Studies of numbers of red cells and of the mean corpuscular volume should be of interest in this regard. It is possible that there may be changes in the size of the red cells, or interference with their release into the peripheral circulation. At any rate, it is obvious that the hematocrit under such circumstances is unreliable as an index to any relative degree of change in the total plasma or red cell volume.

Table 1 (B) shows the reaction of dog 36-94 to the injection. This experiment differed from the others in that plasma volume determinations were made on the day following injection of the acacia solution. At this time there was an increase of plasma volume from 828 to 923 cc., enough to be considered significant. Plasma protein concentration fell from 6.6 to 4.7 and there was a definite decrease in the amount of total circulating protein, amounting to about 10 gm., or approximately 20 per cent. This was not associated with an increase of the urinary nitrogen. On the 3rd day there was an increase in plasma volume of a little over 200 cc. or about 25 per cent over the basal. The

plasma protein concentration diminished from a basal average of 6.60 to 4.53, or about 31 per cent. The total circulating protein in 3 days decreased from 54 gm. to 48 gm. On the 14th day there was an increase of 0.7 gm. of urinary nitrogen, which would be equivalent to about 4 gm. of protein. At this time, the total circulating protein was increasing. Unfortunately, later urine specimens were contaminated with fecal material. This dog also illustrates two points which have been mentioned before, namely the disproportion between plasma

TABLE 2

Single Injection of Gum Acacia

Dog 36-100.

Day	Plasma protein					Hematocrit plasma
	Concentration	Albumin	Globulin	A/G ratio	Fibrinogen	
	gm. per cent	*gm. per cent*	*gm. per cent*		*mg. per cent*	*per cent*
Basal	6.85	3.82	3.03	1.5	307	47.8
1	4.37	2.43	1.94	1.3	176	49.8
2	4.19	2.27	1.92	1.2	204	51.3
3	4.15	2.25	1.90	1.2	82	52.8
4	3.98	—	—	—	109	53.7
5	4.41	2.55	1.86	1.4	119	55.3
7	4.45	2.63	1.83	1.4	118	57.0
9	4.93	2.82	2.11	1.3	125	53.8
11	4.99	2.68	2.31	1.2	154	53.0
13	6.14	—	—	—	317	52.4
20	6.34	3.33	3.01	1.1	286	51.0
27	6.64	3.79	2.86	1.3	250	49.9

per cent and plasma volume, and ultimate diminution of plasma and red cell volume.

Table 2, dog 36-100, represents an animal in which an attempt was made to correlate fibrinogen concentration with plasma protein concentration. It is of interest to note that although the general reaction is the same, the ratio between the two substances is inconstant. For example the fibrinogen reached the lowest level of 82 on the 3rd day, whereas the minimum protein concentration was attained on the 4th day. Likewise, between the 1st and 2nd days, plasma protein concentration decreased, whereas fibrinogen concentration increased. These

changes are not great, but indicate a trend which, it will be seen later, occurs to a greater degree in dogs receiving frequent injections. There was a tendency for the albumin-globulin ratio to decrease a trifle. This same tendency is noted in the following animal, and at the present, its significance if any, is not known.

TABLE 3

Single Injection of Gum Acacia

Dog 35-151.

Day	Plasma protein						Blood plasma volume	R. B. C. volume	Total blood volume	Hematocrit plasma	Urine (48 hrs.)	
	Concentration	Total in circulation	Fibrinogen	A/G ratio	Albumin	Globulin					Volume	Nitrogen
	gm. per cent	*gm.*	*mg. per cent*		*gm. per cent*	*gm. per cent*	*cc.*	*cc.*	*cc.*	*per cent*	*cc.*	*gm.*
Basal	6.04	26.0	337	1.3	3.39	2.65	437	521	958	46.4	538	4.40
1	4.41		248	1.0	2.24	2.16				54.6		
2	4.31		111	1.3	2.43	1.88				52.4	630	4.98
3	4.34	22.4	174	1.1	2.28	2.06	516	509	1025	52.9		
4	4.05		173	1.2	2.24	1.81				55.0	280	3.66
5*	4.57		169	1.2	2.48	2.09				52.7		
6*	4.52		161	1.2	2.45	2.07				53.4	340	4.36
7*	4.69		227	1.2	2.60	2.09				52.6		
9*	4.30	17.0	293	1.3	2.39	1.91	395	286	681	58.0	360	4.14
11†	4.81		406	1.1	2.47	2.34				54.3	266	4.29
14	4.89		396	1.0	2.48	2.41				56.7	266	4.44
18	4.98		338	1.0	2.45	2.53				60.3	320	6.20
22	5.84		359	1.1	3.07	2.77				54.7		
29	5.69		242	1.2	3.14	2.55				56.2		
37	5.89		259	1.2	3.23	2.66				51.9		

* Refused part of diet.

† Diet changed.

Table 3, dog 35-151, shows essentially the same changes noted in the other animals in this group. The change in plasma and red cell volume shrinkage on the 9th day was more marked than that noted in other animals. Changes in fibrinogen concentration were somewhat less marked. On the 1st and 2nd days there were slight in-

creases in urinary nitrogen, followed up to the 11th day by decreases. During this interval, however, the animal was not eating well, and the

TABLE 4

Repeated Injections of Gum Acacia

Dog 32-8.

Date	Plasma protein		Hematocrit plasma	Weight	Acacia given
	Concentration	Fibrinogen			
	gm. per cent	*mg. per cent*	*per cent*	*kg.*	*gm.*
Apr. 13	6.69	217	44.7		33
14	4.43	246	54.2	15.5	
15	4.69		55.0		
16	5.11		55.2		
17	5.03	251	55.9		
18	5.28		52.5		
20	5.68	167	51.2		8
21	4.80		56.5	15.4	
22	5.16	212	52.5		
24	5.56		53.9		16
26	5.12	271	54.8		
28	5.20		54.2	15.5	18
29	3.98	195	59.2		
May 2	4.59	168	53.3		
4	4.91	138	53.8		16
5	4.04	150	56.5	15.3	
6	4.58	142	56.3		
7	4.62	143	54.3		
10	4.98	174	50.6		30
11	3.49	110	58.4		
12	4.00	106	58.2	15.4	
13	4.19	99	56.0		
14	4.38	97	55.6		30
15	3.46	76	59.5		
16	3.48	77	58.8		18
17	3.44	73	61.1		18
18	3.03	59	61.6		18
19	2.73	30	63.2	14.9*	30
20†	2.48	61	61.9		

* Animal consumed all of diet during course of experiment.

† Dog sacrificed (gas anesthesia).

diet was changed to one containing more protein, which lessens the significance of the urinary findings.

Table 4 shows the record of a normal dog (32-8) which received frequent injections of a 6 per cent gum solution. The first injection contained the same amount of acacia per pound as that given the previous four dogs. The amount of fluid injected, though, was twice

TABLE 5

Repeated Injections of Gum Acacia

Dog 36-94.

Date	Plasma protein: Concentration	Plasma protein: Albumin	Plasma protein: Globulin	Plasma protein: A/G ratio	Plasma protein: Fibrinogen	Hematocrit plasma	Weight	Food consumed	Acacia given
	gm. per cent	*gm. per cent*	*gm. per cent*		*mg. per cent*	*per cent*	*kg.*	*per cent*	*gm.*
June 3	6.65	4.19	2.46	1.7	181	45.2	24.1	100	52
10	5.37	3.40	1.97	1.7	144	46.5	24.1	95	26
17	4.66	3.55	1.11	3.2	75	50.5	23.5	100	26
24	4.51	3.24	1.27	2.6	75	51.9	24.1	90	13
July 1	4.97	3.70	1.27	2.9	94	54.0	23.3	95	13
8	5.16	3.73	1.33	2.8	91	54.9	23.5	100	26
15	4.95	3.61	1.34	2.7	—	54.8	23.2	100	25
23	4.84	—	—	—	109	57.4	23.3	100	25
29	5.13	3.65	1.48	2.5	113	55.0	22.9	100	35
Aug. 5	4.83	3.28	1.55	2.1	62	58.6	23.1	100	35
12	4.61	3.30	1.31	2.5	98	58.7	23.5	100	35
19	4.58	3.11	1.47	2.1	91	58.8	23.0	100	20
26	4.74	3.23	1.51	2.1	96	58.2	22.8	100	20
Sept. 2	4.88	3.23	1.64	2.0	125	57.5	22.8	100	20
9	4.94	3.22	1.72	1.9	127	57.0	22.5	100	35
16	4.64	3.07	1.57	2.0	97	55.2	22.7	90	25
23	4.64	2.88	1.76	1.6	118	57.2	21.9	90	40
30	4.36	2.56	1.79	1.4	77	58.0	21.9	80	40
Oct. 7	4.14	2.86	1.28	2.2	74	55.9	21.6	80	40
14	3.94	2.78	1.16	2.4	67	56.5	20.9	70	40
21	3.64	2.38	1.26	1.9	63	56.8	20.1	50	40
28	3.43	2.15	1.28	1.7	84	61.1	19.8	50	40
Nov. 4	3.13	1.92	1.21	1.6	76	61.9	19.1	40	0
11	3.48	2.41	1.07	2.3	101	58.9	18.7	30	0

as large as that given the former animals. It is of interest that the lowest point in plasma protein concentration was reached on the 1st day following injection as compared to the 3rd and 4th days in the other dogs. The fibrinogen did not show the marked variation pre-

viously described. It seems probable that the concentration of acacia and the total amount of fluid given are responsible for the variation exhibited in this animal. Finally, after several injections at relatively frequent intervals, it was possible to reduce the plasma protein concentration to below 3 gm. per cent. The fibrinogen in the meantime had been below 100 mg. per cent for a week, preceding the time the animal was sacrificed. Throughout the course of this experiment which lasted a little over a month, the dog ate well and showed no untoward clinical signs until the last few days when there was slight edema of one leg. There was loss of weight of a little over a pound during the period.

In order to produce a longer period of low plasma protein concentration, another normal dog (36-94, *Table 5*) was given weekly injections of 12 per cent gum acacia, the dose from week to week being graduated according to the dog's reaction during that period. This dog had previously had a single acacia injection. It has been possible to maintain the plasma protein concentration below 4.5 gm. per cent for a period of 9 weeks. The animal showed a steady weight loss until injections were stopped. In a period of 5 months about 660 gm. of gum acacia were introduced into the animal. The fibrinogen maintained a constant low level from the start of the procedure. It is of interest to note that for the most part fibrinogen and plasma protein concentration closely paralleled each other, fibrinogen changes being more marked, however, than plasma protein deviations. The albumin-globulin ratio in this animal had a tendency to be higher than normal, as contrasted with an opposite tendency in dogs receiving single injections. This was due to a relatively greater fall in albumin concentration, although there was a decrease in the concentration of both albumin and globulin.

Clinical Histories

Dog 32-8. Female coach-bull in good condition. Had been previously used in anemia colony. Was given a diet of meat scraps. On Apr. 13, 1937, the first injection of 6 per cent gum acacia solution was given. Thereafter over a period of 38 days, ten intravenous doses of varying amounts of gum acacia solution were given. No untoward clinical signs were noted until 1 month after the initial injection, at which time the animal had received a total of 151 gm. of acacia. At this time bleeding from the needle puncture wound was sustained, and pressure

had to be applied to the vein for some time until it was stopped. The plasma protein concentration was 3.48 gm. per cent, the fibrinogen concentration 77 mg. per cent. On May 20, slight pitting edema of the right leg was noted. The plasma protein concentration at this time was 2.48. The following day the dog was killed with gas and postmortem examination was carried out immediately. At no time was there evidence of jaundiced plasma. The animal ate well at all times and never appeared to be sick.

Autopsy.—An obese dog with moderate pitting edema of hind legs. Bleeding from neck puncture wound which had been made just before death. The peritoneal cavity contains 50 to 60 cc. of clear watery fluid. The pericardial cavity is filled with similar fluid. The blood runs freely from vessels and heart and after 10 to 20 minutes forms rather soft, semi-elastic light red clots. Heart shows a few small reddish granulations along the free margins of the mitral leaflets. Lungs are negative. Spleen is rubbery and large. The cut surface is deep red and large diffuse gray foci, apparently Malpighian bodies, are readily seen. Gastro-intestinal tract and pancreas are essentially negative.

Liver weighs 810 gm. It is swollen and tense; the capsule is smooth, glistening, and the liver parenchyma beneath shows large confluent interlacing grayish colored lobulations. The organ tends to be friable in consistency and cuts with ease. The cut margins evert and bulge. The very congested red color of the cut surface appears limited to small central areas that tend to be obscured by coalescing large dull gray peripheral zones. The main bile ducts and vessels are negative.

Suprarenals and genito-urinary tract are negative. Main arteries and veins are negative. There is no lymphatic enlargement. The subcutaneous tissues of the neck show extravasated bloody fluid. Bone marrow and brain are not remarkable.

Microscopic Examination.—*Heart* negative. *Lungs:* Numerous large mononuclear cells with abundant clear cytoplasm are present in many alveoli.

Liver: All the polygonal cells are greatly swollen and have a foamy appearance due to the presence in the cytoplasm of numerous small vacuoles (Fig. A). Many of the nuclei are pyknotic and irregularly stellate in shape. The majority of the Kupffer cells appear unaltered but some have thick blunt clear cytoplasmic processes. The intracellular material does not stain with Sudan III (fat) nor Best's carmine (glycogen). This appearance is quite uniform, but there is a suggestion of slightly greater involvement in the mid-zonal regions and in the cells about portal areas. The bile ducts are essentially negative.

Spleen: The pulp, in scattered areas, shows many large clear cells filled with vacuoles similar to those seen in the liver cells. *Kidney:* The epithelium of the convoluted tubules is not remarkable. The epithelium of most of the collecting tubules appears to be finely vacuolated. An occasional glomerular tuft in both kidneys is partially or completely composed of large, irregular, vacuolated, clear cells. Scattered small foci of lymphocytes, plasma cells, and neutrophils are present.

Anatomical Diagnosis.—Congestion of, and infiltration of acacia into liver cells. Infiltration of acacia into epithelium of collecting renal tubules. Pitting edema of lower posterior extremities. Fluid in peritoneal and pericardial cavities. Acute (early) endocarditis.

Dog 36-94. A short-haired black and white spotted male mongrel weighing 19.3 kg. Vaccinated against distemper. On Dec. 31, 1936, put on hamburger-

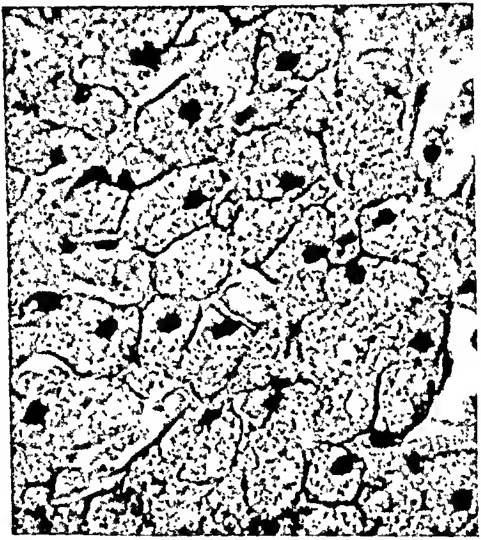

FIG. A. Photomicrograph of section of liver of a dog (32-8, Table 4) which had [illegible] of gum acacia solution. Swelling and vacuolization of [illegible] ×650.

potato basal diet containing 2 gm. protein per kilo body weight. Samples of blood were taken over a period of 9 days. On Jan. 9, 1937, was given 42 gm. of gum acacia made up to a 12 per cent solution with Locke's solution. Dog stood injection well, did not vomit. The animal was studied for a period of 16 days. It was then returned to the animal house and kept on kennel diet until June 3, 1937, when potato-hamburger diet was instituted. On the same day 52 gm. of gum

acacia solution was given. (In the meantime the weight had increased 4.8 kg.) From that time until Oct. 28, 1937, the dog was given an intravenous injection of gum acacia every 7 days, blood being removed for analysis previous to each injection. The dog gradually lost weight, and ate less and less food. At the present the dog has not received acacia injections for 3 weeks, is eating all food, and is gaining weight. Plasma protein and fibrinogen have increased a little but are still far below normal limits.

Dog 35-151. Short-haired male mongrel, vaccinated against distemper. The animal had been on kennel diet for some months previous to start of experiment. On Aug. 23, 1937, it was put in a metabolism cage, given potato-hamburger diet. Three basal determinations were made during a period of 4 days. On Aug. 27 the dog was given a single injection of gum acacia, 1 gm. per pound weight. The dog was studied for a period of 37 days. From the 4th to the 11th days the animal did not eat well, so the diet was changed to one of table scraps. Except for this episode, the animal at all times remained in excellent clinical condition.

Dog 36-100. A long-haired female brown mongrel vaccinated against distemper. Put in a metabolism cage Aug. 23, 1937, and given potato-hamburger diet. The animal was given a single injection of gum acacia solution on Aug. 27. It was studied for 15 days following this procedure. At all times the animal ate well, no untoward clinical signs were noted.

Dog 36-21. A long-haired mongrel female collie vaccinated against distemper. On Oct. 21, 1936, the present study was started. On Oct. 30 a single injection of gum acacia solution was given. The animal was studied for 10 days following this procedure. At all times it remained in excellent clinical condition.

DISCUSSION

The question arises as to what becomes of the protein which has disappeared from the blood. As far as studies of the urine are concerned, although they are not entirely satisfactory we have not been able to demonstrate enough of an excess of nitrogen to account for that which has disappeared from the blood stream. Is the plasma protein then deposited in storage places in the body? If we assume that the osmotic pressure of the blood plasma of a normal animal cannot be altered for long, and that the circulating plasma volume cannot be increased above a certain point, it is apparent that, if a colloid substance be added to the plasma in large amounts, some colloid must leave the blood stream. It is quite probable that the plasma proteins

are more rapidly withdrawn than acacia. The initial plasma protein diminution following gum acacia injection may represent merely the colloid adjustment necessary to keep the osmotic pressure of the plasma constant. The plasma protein would then return to the circulating plasma as quickly as the acacia could be removed. The work of Stanbury, Warweg, and Amberson (12) in which both plasma protein and acacia determinations were made following total plasmapheresis may be consistent with this thesis, for they have shown that the plasma protein concentration in the blood rapidly rises as that of acacia falls.

In spite of the difference of method, our work and theirs may be comparable. The total amounts of acacia remaining in the circulation following total plasmapheresis were not over twice as much as those given by us in single injections. It seems not unlikely that the organism could handle the two quantities with almost equal facility, since the liver appears to be the organ most involved, and its reserve is known to be great. If this is so, plasma protein levels in the two cases, although quite unlike at first, should, at a given time following injection, be similar. In the case of *total plasmapheresis*, plasma protein must be poured into the circulation starting from a concentration of almost zero as the acacia is removed. In *acacia injection*, the protein must be first withdrawn from the circulation in order to maintain the osmotic pressure and then replaced as the acacia is removed. In the total plasmapheresis experiments normal plasma protein levels were reached within 10 days after substitution of acacia for plasma. In one of our animals (36-21) the curve of the plasma protein concentration from the 5th through the 10th days was quite similar to this. In three out of four of our dogs normal levels were not reached in over 2 weeks. The 5th day, however, is of considerable significance. The plasma protein concentrations of all the dogs in both groups of experiments were quite similar ranging between 4 and 5 gm. per cent.

Another possible explanation of the diminution of plasma protein following injection of gum acacia must be considered. Andersch and Gibson (4) have shown that the liver is responsible in great part for the removal of the acacia from the blood. There is considerable evidence (1, 2, 9) that the liver constitutes the chief warehouse for

reserve protein. If the liver is substantially filled with acacia, it is conceivable that this may be at the expense, in part at least, of reserve protein. It has been further pointed out that there exists a constant utilization or *wear and tear* of plasma protein that may amount to as much as several grams of protein a day (8). In a normal animal, if there should be reduction in production of plasma protein, one would expect to find a decrease in the total amount of circulating plasma protein equivalent to the amount utilized. It is probable that acacia might interfere with the liver function and production of plasma protein to this extent.

Reticulo-endothelial structures have been implicated by various investigators as possible sites of plasma protein formation. While at present we have no specific method for positive identification of acacia histologically, in Eck fistula dogs which have received intravenous gum acacia[2] and in dogs receiving liver poisons such as carbon tetrachloride along with gum acacia,[2] we have noted marked depositions of what is apparently the gum in the spleen and lymph nodes. In the normal dog receiving the gum which came to autopsy in the present study, by far the most extensive and severe histological involvement was that of the liver (Fig. A). This finding fits in with the work of Andersch and Gibson (4) who showed that considerable quantities of gum acacia were deposited in, and excreted by, the liver. Evidence of liver damage is indicated by the marked fibrinogen reduction, for it is generally conceded that the liver alone is the source of this substance (11). On the basis of these observations, regardless of other factors that may enter into the picture, we cannot dismiss the idea that the liver function is markedly disturbed following such injections. By the use of such technique, it should be possible to investigate other phases of liver function. Such studies might throw added light on both the function of the liver and on the acacia-protein phenomenon itself.

SUMMARY

Lowered plasma protein concentration following single injections of gum acacia in the dog is due in some part to dilution, and in greater

[2] Unpublished data.

part to actual decrease in total circulating protein. The maximum decrease in the total circulating protein does not take place at the same time as the maximum decrease in concentration. Fluctuations in fibrinogen concentration are marked, and are not necessarily proportional to changes in plasma protein concentration. Plasma protein concentration returns to normal limits within 10 to 21 days after the injection, at which time total circulating protein and plasma volume are lower than normal. Loss of protein cannot be accounted for by increase in urinary nitrogen.

It is possible to maintain dogs at low levels of plasma protein concentration for several weeks by repeated injections of gum acacia solution. Anatomical changes following such a procedure in a normal dog are most conspicuous in the liver (Fig. A). These observations further implicate the liver as a source of plasma protein.

Two mechanisms for the diminution of plasma protein following gum injection are suggested. One of these is based on the possibility that the liver cells being engorged with gum acacia are not able to produce the necessary amount of plasma protein to supply the normal demand. The other possibility is that with the injection of the gum, since there is obviously a greatly increased amount of colloid in the blood, the more readily removable colloid, *i.e.* plasma protein, is taken out of the blood stream, in an attempt to return plasma volume and colloid osmotic pressure to the normal limits. It is probable that both of these mechanisms are involved.

Injection of gum acacia is suggested as a technique for further study of disturbed liver function.

It must be obvious that clinical use of gum acacia for intravenous injection is not without danger.

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IMMUNOLOGICAL STUDIES OF A HEAT-STABLE SUBSTANCE ISOLATED FROM TISSUES INFECTED WITH VACCINE VIRUS

By ROBERT F. PARKER, M.D.

(From the Department of Medicine, Western Reserve University, School of Medicine, Cleveland)

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There are present in extracts of vaccine virus-infected tissues soluble substances which precipitate specifically when mixed under the proper conditions with antivaccinal immune serum. At least one of these is destroyed by boiling in aqueous solution and it has therefore been designated as the "L," or heat-labile substance. The other, resistant to boiling in neutral solution, is designated as the stable ("S") substance (1). Our recent studies have been concerned chiefly with the latter material and in a previous communication (2) we have reported the isolation from tissues infected with vaccine virus of a heat-stable, alcohol-soluble substance, presumably a pure protein, which precipitates specifically with antivaccinal serum. It was not determined whether this material was antigenic.

It has been stated that the heat-stable soluble specific substances of vaccine virus were not antigenic in rabbits. Smith (3) used a partially purified preparation, made by boiling an extract of virus-infected testicles at pH 5.5, 8.0, and 7.0 successively, and removing the coagulated protein at each step. He reported that the injection of this material into rabbits was followed by no detectable antibody response and suggested that in the manner of its action the substance resembled the bacterial haptenes. Ch'en (4) employed the technic of Smith as a preliminary step and then purified the active substance further by repeated precipitations with alcohol-ether and with alcohol and dialysis against tap water. He obtained a substance which he considers to be a polysaccharide and reported that it was non-antigenic in rabbits. On the other hand Craigie (5) has made preparations of the heat-stable substance which he partially purified by precipitation at pH 4.6. He states that these substances elicit a prompt antibody response when injected into rabbits which have previously been vaccinated. Since these animals have, or have once had, antibodies against the substance the response is a secondary one.

Craigie does not report on the response of normal rabbits to injection with his heat-stable substance.

The work of Craigie suggests that the heat-stable substance with which he is working is antigenic since it is capable of stimulating a prompt secondary formation of antibodies in already immune rabbits. But the failure of heated, serologically active extracts to elicit antibody formation in the experiments of Smith and those of Ch'en would seem to indicate that the substances remaining in solution were of the nature of bacterial haptenes. The difficulty may be more apparent than real for it is well known that appreciable quantities of an antigen are required to initiate the formation of antibodies, while much smaller amounts will suffice to produce a rapid rise of antibody titer in the serum of animals once immunized but in whose serum antibodies may be no longer detectable.

EXPERIMENTAL

Since we had been able to isolate from vaccine virus-infected tissues a heat-stable substance, presumably a protein, which was evidently specifically related to vaccinia, it became of importance to determine whether or not it was antigenic. In this communication we describe the response of rabbits to repeated injection with a purified preparation of the material, and report the results of experiments designed to determine the relation of this to other antigens of vaccine virus. Experiments are also described which were made in an effort to learn the relationship between antibodies against this substance and immunity to vaccinia.

Materials and Methods

1. Preparation of Heat-Stable Substance.—The heat-stable precipitating substance was prepared as described in our previous communication (2). The method may be summarized as follows: An aqueous extract of vaccine virus-infected tissue was prepared and passed through a Seitz filter; the filtrate was incubated at 37°C. for 5 days, then boiled for 5 minutes and the coagulum which formed was discarded. The precipitate from 25 per cent saturation with ammonium sulfate was discarded. The concentration of ammonium sulfate was increased to 50 per cent of saturation, and the resulting precipitate was collected and dissolved in buffer solution (pH 7.0). After dialysis for 2 days against running tap water the material was centrifuged and the sediment of water-insoluble material discarded.

The reaction of the solution was adjusted to pH 4.6 and the precipitate collected. This was dissolved in buffer solution at pH 7.0 and the active substance precipitated by addition of 9 volumes of neutral absolute alcohol. The precipitate was dissolved in water and the alcohol was removed by dialysis. For reasons of economy most of the preparations were made from extracts of dermal vaccine virus.

2. *Pure Anti-L Serum.*—Serum against the labile substances of vaccine virus was prepared from antivaccinal immune serum containing both L and S precipitins. Antibodies against the S substances were removed from the serum by absorption with S antigen.

3. *Titration of Antibody Content of Sera.*—The methods of conducting agglutination, precipitation, and complement fixation reactions have already been described (6). Neutralization tests were carried out as follows: Serial tenfold dilutions of virus (in the form of washed elementary body suspension) were prepared in Locke solution. To 0.3 cc. of each dilution an equal volume of the serum to be tested was added. After a brief incubation 0.25 cc. of each mixture was inoculated intradermally in a rabbit, and the animal observed daily for the appearance of lesions. In order to avoid possible individual variations in susceptibility to vaccinal infection between animals, all samples of serum from a single experimental animal were tested in one rabbit.

4. *Tests of Resistance to Vaccinal Infection.*—After completion of the series of inoculations of antigen and collection of serum, the animals used were tested for resistance to infection with vaccine virus. For this purpose they were inoculated intradermally with serial dilutions of vaccine virus in the form of washed elementary bodies of vaccinia.

5. *Housing of Animals.*—In order to guard against accidental infection with vaccinia of animals which were receiving injections of the pure S substance, arrangements were made to keep them in separate animal quarters. In this way they were segregated from animals infected with vaccinia and were cared for by attendants who had no contact with vaccine virus.

Determinations of Antigenicity in Rabbits

Since the heat-stable substance which we had prepared was derived from rabbits, it was deemed advisable to prepare antisera in the same animal species in order to avoid confusion due to non-specific reactions.

A solution of the pure substance was prepared and distributed in ampoules, which were hermetically sealed. These were submerged in boiling water for 90 minutes in order to sterilize the contents and to destroy any L antigen which might be present. 3 rabbits were given a series of intraperitoneal inoculations of 1, 2, 4, 4, and 4 cc. of the solution at weekly intervals (the solution had a precipitating titer of 1:320). 2 weeks after the last injection the rabbits were bled; the serum was separated and tested for the presence of antibodies.

The serum obtained from the rabbits following the series of injections contained antibodies directed specifically against the heat-stable substance. Inspection of Table I, in which the results of titrations are recorded, reveals that the sera precipitated in dilutions of 1:8 to 1:32 with extracts of dermal vaccine virus and agglutinated the washed elementary bodies of vaccinia in dilutions of 1:128 to 1:256. That the reactions did not involve products of bacterial contamination is demonstrated by the positive complement fixation reactions. The antigen employed in these tests was vaccine virus, cultivated in a chick embryo-Tyrode solution medium according to the method of Rivers.

TABLE I

Titration of Serum Obtained from Rabbits before and after Injection with S Substance

Reaction	Antigen	Serum					
		360		361		492	
		Before	After	Before	After	Before	After
Precipitation	Extract dermal vaccine virus	–	1:32	–	1:32	–	1:8
Agglutination	Washed elementary bodies	–	1:128	–	1:256	–	
Complement fixation	Cultured vaccine virus	–	1:16	–	1:8	–	

Absorption Experiments

That the antibodies engendered in response to injections of the stable antigen were specific for vaccine virus has been shown. That they were specifically directed against the stable antigen was shown as follows:

1. Absorption of Serum with Stable Antigen.—A portion of the serum was absorbed with a solution of the heat-stable antigen by the following technic. The ratio of optimal precipitation was determined according to the method of Dean and Webb. Serum and antigen were mixed in the indicated proportion, incubated at 50°C. for 1 hour, and centrifuged at 4000 R.P.M. for 1 hour in the angle centrifuge. The supernatant fluid was removed, and after remaining in the ice box overnight was centrifuged. The clear solution resulting failed to precipitate when mixed either with stable antigen or with a fresh filtrate containing both L and S antigens. The protocol of such an experiment is given in Table II.

This experiment shows that absorption of the serum with stable antigen was capable of removing all of the precipitins against S antigen,

and that precipitins against L antigen had not appeared in the serum of rabbits injected with S substance.

TABLE II

Absorption of Anti-S Antibodies from Serum of Animals Injected with S Substance

Serum	Antigen	Dilution of serum							
		1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
361 untreated	Fresh extract of dermal virus containing L and S antigens	+	++	++	++	+	−	−	−
	Boiled extract of testicular virus containing S antigen	++	++	++	+	±	−	−	−
	Suspension of heated elementary bodies containing S antigen			±	+	+	±	±	−
361 absorbed with S antigen	Fresh extract of dermal virus containing L and S antigens	−	−	−	−	−	−	−	−
	Boiled extract of testicular virus containing S antigen	−	−	−	−	−	−	−	−
	Suspension of heated elementary bodies containing S antigen	−	−	−	−	−	−	−	−

TABLE III

Absorption of S Antigen from a Mixture of L and S

Antigen solution	Test serum	Antigen dilutions					
		1:2.5	1:5	1:10	1:20	1:40	1:80
Fresh extract of dermal virus containing L and S antigens	L serum*			++	++	+	−
	S serum†			++	++	−	−
	Normal serum			−	−	−	−
Fresh extract absorbed with anti-S serum	L serum	++++	++	++	+	−	−
	S serum	−	−	−	−	−	−
	Normal serum	−	−	−	−	−	−

* S antibodies removed by absorption.

† Prepared against pure S substance.

2. *Absorption of Antigen with Pure Serum.*—A filtrate of dermal vaccine virus was prepared which contained both L and S antigens. The optimal ratio was determined for precipitation with anti-S serum, and to a quantity of filtrate the calculated amount of serum was added. The mixture was incubated at 50°C. for

30 minutes, held in room temperature for 2 hours, and centrifuged at 4000 R.P.M. for 1 hour in the angle centrifuge. After remaining in the ice chest overnight, the solution was again centrifuged and the clear solution tested for the presence of the precipitating substance. The results are shown in Table III.

As is recorded in Table III, the crude skin filtrate which had been absorbed with our serum failed to yield a precipitate when more of the same serum was added. The addition of serum containing antibodies against the heat-labile substances gave a precipitate in almost as high dilutions of filtrate as before absorption. The anti-S serum had removed the heat-stable substances specifically, while leaving the heat-labile substances in solution.

TABLE IV

Neutralization Tests with Serum of Animals Injected with Pure S Substance

Serum	Highest dilution of virus causing lesion when mixed with undiluted serum
360 (anti-S)	10^{-4}, 10^{-4}
361 (anti-S)	10^{-4}, 10^{-4}
Normal control	10^{-5}, 10^{-6}
Immune control	$>10^{-1}$,* $>10^{-1}$

* No lesion produced by virus diluted 10^{-1} when mixed with immune serum.

Relation of Antibodies against the Heat-Stable Antigen to Vaccinal Immunity

Since it had been demonstrated that injection of the S antigen into rabbits caused antibodies against it to appear in the serum of these animals, it became of importance to determine whether this anti-S serum was capable of neutralizing the infective capacity of vaccine virus, and whether the animals themselves were resistant to inoculation with this pathogenic agent. This was determined in the following manner.

Neutralization tests were carried out as described above under Methods, as was also the determination of resistance of the rabbits to infection with vaccinia. The results of the neutralization tests are collected in Table IV.

Inspection of Table IV reveals the fact that the serum of the animals injected with S substance apparently neutralized small amounts of virus. The difference, however, in the effect on vaccine virus between the normal and anti-S sera is very slight and it may be

questioned whether it is sufficient to be taken as an indication of actual immunity. Furthermore, inoculation of minimal amounts of virus gave rise to infection in the tested animals. The lesions produced by a given dilution of virus appeared to be of the same severity in all the animals.

SUMMARY AND CONCLUSIONS

We have shown that it is possible to obtain from extracts of vaccine virus-infected tissues a substance or substances, apparently protein, which are serologically active, and which are specifically related to vaccinal infection. The present investigation is concerned with a study of their immunological reactions. Their intraperitoneal injection in rabbits is followed by the appearance in the serum of these animals of antibodies directed specifically against them. The precipitating capacities of this serum are entirely removed after addition of appropriate quantities of heated vaccine virus extract, indicating that antibodies against only the heat-stable antigens have been produced. Further evidence of the specificity of the antibodies is gained from the reverse experiment, that is, absorption of a virus extract with the serum. We have shown that under suitable conditions the serum will remove S antigen specifically leaving the labile substances in solution. This would apparently indicate that they are serologically distinct although both are vaccinal products.

Serum of animals injected with the S substance and containing antibodies against it in high titer is apparently capable of neutralizing minute amounts of active virus. The animals providing the serum are, however, without demonstrable resistance to vaccinia. The significance of the neutralizing activity of the serum is debatable because it is of a greatly different order of magnitude from that which follows infection with vaccinia.

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TOXEMIA OF PREGNANCY IN THE RABBIT

II. Etiological Considerations with Especial Reference to Hereditary Factors

By HARRY S. N. GREENE, M.D.

(From the Department of Animal and Plant Pathology of The Rockefeller Institute for Medical Research, Princeton, New Jersey)

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A description of the clinical manifestations and pathological lesions of toxemia of pregnancy in the rabbit and evidence indicating an analogy with eclampsia in man were presented in a previous paper (1). Clinical and pathological study suggested that the disorder was of endogenous origin, but a discussion of etiological factors proper was deferred pending presentation of the evidence derived from a study of the incidence.

The object of the present paper is to analyze the incidence of the disorder with respect to pregnancy, age, breed and genetic constitution, and to consider the results of this study together with the clinical and pathological findings as factors of etiological significance. Detailed analysis of the incidence will be limited to a period extending from November, 1935, to December, 1936, during which there was an unprecedented number of cases, but the previous occurrence of the disorder will be described and factors of possible significance in the genesis of the outbreak will be discussed.

Materials and Methods

The material for the present report is based on 72 fatal cases of toxemia which occurred between November, 1935, and December, 1936, supplemented by data obtained from previous observations. The colony in which the disorder occurred and the composition of the population were described in the first paper of this series (1). The origin, integrity and relations of the different pure breeds have also been described in previous publications (2, 3).

Incidence

Toxemia of pregnancy in the rabbit may occur as a rapidly fatal affection or as a comparatively mild, frequently asymptomatic disorder followed by recovery. The detection of non-fatal cases is not always possible, and as the incidence cannot be accurately determined, analytical studies are necessarily limited to the occurrence of fatal cases.

Fatal toxemia of pregnancy was first noted in 1931 and two cases were recorded during that year (Table I). The number of cases gradually increased in following years, but the incidence remained relatively low until in November, 1935, a rapid increase began and continued through December, 1936. The monthly incidence during this outbreak period is presented in Table II. It is of interest, in view of the seasonal variations in the frequency of human eclampsia, that the majority of cases both during this period and in previous years occurred during the late winter and early spring.

TABLE I

The Annual Incidence of Toxemia from 1931 to 1936

Year	Number of cases of toxemia
1931	2
1932	3
1933	7
1934	11
1935	25
1936	55

TABLE II

The Monthly Incidence of Toxemia during the Outbreak Period

	1935		1936											
	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.
Number of cases of toxemia	0	17	8	19	7	4	5	3	0	1	1	2	4	1

72 animals died of the disorder in this outbreak, an incidence in the female population of 11.07 per cent. Cases were not distributed evenly throughout the population, but occurred with greater frequency in certain breeds and families, and in the following paragraphs the incidence during the 13 months' period will

be analyzed with reference to the differential response of various elements of the population.

It should be emphasized from the beginning, however, that all susceptible animals were not affected by the disorder during the period under consideration. A number have since died of toxemia and others will undoubtedly die at future dates. The results obtained from analysis of the incidence are, therefore, not absolute but relative to that period only, and differences in the incidence in various constitutional groups would probably be greater if the period were longer or equalled the life of the population. The occurrence of the disorder in both sporadic and outbreak form suggests that the incidence may be greatly influenced by environmental factors and that under favorable conditions a susceptible animal may live its life unaffected. Therefore, as the only test of susceptibility is death from toxemia, susceptibility studies must necessarily be conducted during periods of high incidence although such periods may include only a short portion of the life of the population.

Relation to Pregnancy

The disorder was not limited to pregnant rabbits, but occurred postpartum and in resting animals. In the present outbreak 59.3 per cent of cases were in pregnant does, 20.8 per cent in postpartum and 19.4 per cent in resting females.

Among pregnant does all cases with one exception occurred during the last week of gestation, usually on the 28th day. The one exception occurred on the 17th day of pregnancy. 5 cases of the disease occurred on the 1st day following delivery and only 2 cases appeared later than a week postpartum.

With reference to deaths among resting females, it is of considerable interest that at autopsy corpora lutea were found in the ovaries. 12 of these animals had recently been mated and were presumably in a condition of pseudopregnancy. In explanation of this term, it should be pointed out that in the rabbit ovulation is induced by copulation, and if fertilization fails to occur, the female may still exhibit all the symptomatic changes of pregnancy. In such animals the temperamental changes associated with pregnancy have frequently been observed and nest building and lactation are not uncommon near the end of the pseudogestation period which is of irregular duration. These manifestations, however, are not limited to infertile mated animals, but are also occasionally noted in unmated animals. In two instances the typical clinical symptoms and pathological lesions of the disorder were found in animals that had not been mated in over 6 months but had been caged in open wire compartments in close proximity to other animals. It seems to be well authenticated that rabbits may ovulate as a result of contact without copulation, and such instances indicate that mere proximity to other animals may be sufficient stimulus to induce ovulation and that pseudopregnancy of this order may be of common occurrence.

The duration of the state of pseudopregnancy in does dying in that condition is difficult to determine inasmuch as many animals had been mated a number of times in the period preceding the occurrence of the disorder. When estimated from the last mating, the period averages 10 days, but if only those cases are considered in which a single mating had been made, the period averages 25 days, and this approximates that found in true pregnancy.

Multiparity.—There were 66 fatal cases of the disorder in multiparous does, an incidence of 10.3 per cent, and only 6 cases, or an incidence of 2.6 per cent, in primiparae. The disorder did not occur in virgin females. This distribution is contrary to that observed in human eclampsia where the frequency is far greater in primiparae than in multiparae. It should be noted, however, that in the rabbit repeated attacks of toxemia occur and the primary attack may be mild and asymptomatic. It is probable that the disorder occurs in primiparous rabbits with far greater frequency than is recognized and that *the higher incidence of fatal cases in multiparae is due to repeated* attacks.

Fertility.—In the routine conduct of the colony, animals are examined for pregnancy on the 10th day after mating and if found non-pregnant, are remated. The fertility of the colony is thus under constant check, and examination of the records of animals that subsequently died of toxemia brings out a point of interest.

The multiparous does had borne from 1 to 10 litters each with an average of 5 litters. 12 of these animals had gone through uncomplicated pregnancies from 1 to 3 months previous to the fatal attack, but the remaining 54 animals had not borne litters for periods ranging from 7 to 15 months. This long interruption was due in part to cessation of breeding during the summer of 1935, but in larger part to infertility for the animals had been mated repeatedly without conception, both before and after the summer interval. A similar history of sterility obtained in the primiparae and in one instance as many as 12 non-fertile matings had been made. The frequent history of a long period of unproductivity followed by resumption of fertility and death from toxemia in the ensuing pregnancy may be of etiological significance.

Age

The age incidence of fatal cases of toxemia varied from 5 to 53 months. On a percentage population basis the incidence was 2.4

per cent among animals less than 1 year old, 17.8 per cent in the group between 1 and 2 years, 15.5 per cent in the group between 2 and 3 years, 22.2 per cent in the group between 3 and 4 years, and 18.1 per cent in the group between 4 and 5 years.

TABLE III

Distribution of Cases of Toxemia in Pure Bred Animals and in Various Hybrid Generations

Breed	Pure bred		First hybrid generation		Second hybrid generation		Backcross hybrid generation		Other hybrid generations		Total hybrids		Total hybrid and pure bred	
	Number of animals	Mortality	Number of animals	Mortality	Number of animals	Mortality	Number of animals	Mortality	Number of animals	Mortality	Number of animals	Mortality	Number of animals	Mortality
		per cent		*per cent*		*per cent*		*per cent*		*per cent*		*per cent*		*per cent*
Belgian	31	3.2	32	3.2	0	0	9	0	54	7.4	95	5.2	126	4.7
Blue Beveren	3	0	3	0	1	0	9	11.1	6	0	19	5.2	22	4.5
Dutch	19	26.3	23	21.7	7	14.2	18	5.5	37	13.5	85	12.9	104	15.3
English	42	14.2	49	6.1	1	0	7	14.2	100	10.0	157	8.9	199	10.0
Himalayan	14	0	19	42.1	2	0	2	0	15	13.3	38	26.3	52	19.2
Havana	31	9.7	46	21.7	4	25.0	3	33.3	59	13.6	112	17.8	143	16.0
Polish	4	50.0	57	33.3	11	9.0	9	0	34	14.7	111	22.5	115	23.4
Rex	2	0	15	26.6	4	25.0	15	20.0	17	5.9	51	17.6	53	17.0
Chinchilla, Lilac, Marten, French Silver, Tan, Sable	15	0	21	0	4	0	7	0	18	0	53	0	68	0
Himalayan-Albino hybrids									115	9.5	115	9.5	115	9.5
Polyhybrids of complex genetics									31	3.2	31	3.2	31	3.2
Total.	154	11.03	181	16.4	23	13.0	82	9.7	209	6.7	496	11.08	650	11.09

The low incidence noted in the youngest group was probably related to parity rather than to age which was apparently of little significance in the determination of susceptibility.

Breed

The distribution of fatal cases of toxemia in the pure bred animals and in the various hybrid generations of each breed is shown in Table III. The hybrids are classified into first, second and backcross generations, and a fourth class designated as other generation hybrids includes all other animals derived from the pure

breeds with the exception of two groups which are listed separately. One of these is composed of polyhybrid animals of extremely complex genetic origin; the other is a group of Himalayan-Albino hybrids that had been inbred since 1918. The Himalayan ancestors of this group were pure bred animals, but the Albinos were a mongrel stock of uncertain origin derived in part from a Dutch-Polish cross. All other hybrids are listed under each of the various breeds from which they were derived and, as this necessarily involves duplication, the total number of animals included in each generation is listed in the final column of the table.

There was no significant difference in the incidence of the disorder in pure bred and hybrid animals; the incidence was 11.03 per cent in pure bred stocks and 11.08 per cent in hybrids. There were, however, marked differences in the distribution of cases in the various pure breeds and in the different hybrid classes and subgroups.

Pure Breeds.—The disorder did not occur in the Beveren, Chinchilla, Himalayan, Lilac, Marten, Rex, Sable, French Silver or Tan breeds. In the remaining breeds the incidence was highest in the Polish and Dutch, intermediate in the English and Havana, and lowest in the Belgian.

A number of breeds were represented by relatively few animals, and the significance of the incidence is doubtful if measured statistically. Other evidence, however, shows that with two exceptions, the pure bred animals present during the outbreak period were truly representative of their breed and the results obtained may be considered as indicative of the relative incidence had larger groups been available. The two exceptions consist of the Himalayan and Rex breeds. Typical cases of toxemia occurred in both of these breeds in years previous to the period under consideration and occurred during the present outbreak in animals derived from repeated backcross matings, and it is assumed that the small pure bred groups present in the colony were not representative.

In view of the wide differences in incidence breed may, therefore, be regarded as a factor of considerable importance in the determination of susceptibility. It is of interest in this connection that the Dutch, Polish and Havana breeds, all of which showed high susceptibility, are racially related and belong to a group which is fundamentally Dutch.

Hybrids.—The incidence in Himalayan and Rex hybrids was high, but otherwise the position of the different hybrid groups was generally comparable with the arrangement of their pure bred ancestors. With the exception of the Himalayan, Rex and Beveren groups, the hybrids derived from breeds in which toxemia did not occur were also un-

affected by the disorder. The position of the Himalayan and Rex breeds in this respect has been referred to above. The single case in the Beveren hybrids occurred in an animal obtained from a backcross mating of an Havana-Beveren hybrid, and the factors determining susceptibility may have been carried over from the Havana breed.

The relatively low frequency of toxemia among Himalayan-Albino hybrids is of interest in view of the fact that in former years the highest incidence occurred in this group. It is probable, however, that the majority of susceptible animals had previously died of the disorder, and that the population present during the outbreak was to a large extent composed of non-susceptible survivors.

On a basis of the incidence in the various pure breeds and their hybrid derivatives, the arrangement of breeds as factors in the determination of susceptibility stands Polish, Himalayan, Rex, Havana, Dutch, English, Belgian, Beveren in order of decreasing importance. Additional and more detailed information of the relative importance of breed in this connection may be obtained by further analysis of the first hybrid generation and a classification of animals with reference to both parental lines.

First Generation Hybrids.—41.6 per cent of all cases of toxemia occurred in first generation hybrids and the incidence in this group was 16.4 per cent compared with an incidence of 8.9 per cent in the remainder of the colony.

There were marked differences in mortality in the F_1 hybrids derived from different breeds, but the position and arrangement of the various groups in an incidence scale is in general agreement with that noted in total hybrids in the previous table.

No cases occurred in the first generation progeny of the Beveren, Chinchilla, Sable, French Silver or Tan breeds. The incidence was greatest in the groups derived from the Himalayan, Polish, Rex, Havana and Dutch breeds and least in those derived from the English and Belgian breeds.

The F_1 generation hybrids may be subdivided into two classes for more detailed examination. One class which was derived from the mating of pure bred animals with unrelated hybrids and in a strict sense is not a true F_1 generation, contained 86 animals of which 8 died of toxemia. The other class was derived from the crossing of pure breeds and contained 100 animals of which 22 died of the disorder. Closer analysis of the first class with its more complex breed relationships fails to give further information concerning the influence of breed on the incidence of the disorder. The animals of the second class, however, may be analyzed with

reference to both parental lines as in Table IV, and by comparing the incidence in vertical and horizontal planes the relative influence of each breed on the susceptibility of the F_1 generation may be gauged.

21 separate groups of first generation hybrids result from such a classification, but cases of toxemia occurred in only 8 of these groups, namely, the Dutch, English, Himalayan and Havana-Polish hybrids, the Belgian, English and Rex-Havana hybrids, and the Himalayan-Rex hybrids. These groups were represented by 60 animals and the remaining 13 groups composed of 40 animals were without mortality. It is significant that 22 cases of the disorder or more than 30 per cent of the total incidence occurred in approximately one-tenth of the total female population of the colony.

TABLE IV

Distribution of Cases of Toxemia in First Generation Hybrids
(Mortality Per Cent)

	B	D	E	H	HA	P	R	BA	C	M	S	SA	T
B			0		33.3	0							
D			0		0	33.3							0
E	0	0			16.6	33.3		0	0			0	0
H						46.6	25.0						
HA	33.3	0	16.6			35.7	60.0					0	0
P	0	33.3	33.3	46.6	35.7								
R				25.0	50.0								
BA			0										
C			0										0
M											0		
S										0			
SA			0		0								
T		0	0		0				0				

B = Belgian; D = Dutch; E = English; H = Himalayan; HA = Havana; P = Polish; R = Rex; BA = Blue Beveren; C = Chinchilla; M = Marten; S = Sable; SA = French Silver; T = Tan.

The 8 groups were derived from the crossing of 7 pure breeds, all of which with the exception of the Rex and Himalayan breeds contained cases of toxemia. On the other hand, the disease did not occur in the 13 groups derived from the crossing of non-susceptible breeds and of non-susceptible with susceptible breeds.

Examination of the table shows that the various first generation hybrids shared the susceptibility differences exhibited by the parental pure breeds and that, with minor variations in position, the incidence scale of the first generation agrees with that of the pure breeds. Moreover, with the further exception of the Rex and Himalayan hybrids, the proportion of groups affected by the disease in the different first generations varied with the position of the parental breed in the incidence

scale. Thus, cases of toxemia occurred in 4 of the 5 Polish and Havana groups, in 2 of the 4 English groups, and in 1 of the 3 Dutch and Belgian groups. The occurrence of the disorder in both of the 2 Himalayan and Rex groups is considered as additional evidence that the pure stocks of these breeds present during the outbreak were not representative. Evidence that breeds higher in the incidence scale exerted a greater influence on their first generation hybrids is brought out by a comparison of the incidence in the different groups derived from the same breed. For example, the incidence was greater in pure bred Polish than in pure bred Havanas, and the susceptibility of Polish-English and Dutch hybrids was greater than that of Havana-English and Dutch hybrids. In connection with the high susceptibility of Polish hybrids, it should be noted that the incidence of the disorder was greater in the first generation than in the parent breed in all instances with the significant exception of the Polish breed in which the incidence was greater than in any of its first generation hybrids.

Other Generation Hybrids.—The highest incidence in all classes of animals occurred in the first generation hybrids, but in the second and backcross generations the frequency of cases was decreased and approached that noted in the pure bred population.

The number of animals in the second and backcross generations was small and the position of the hybrid groups was not constantly related to that of the breeds from which they were derived. The arrangement of groups in other hybrid generations and in total hybrids, however, was remarkably constant and was in general agreement with that of the parent pure breeds.

It is evident from the data presented above that factors influencing susceptibility were closely associated with breed or race and that the differing susceptibility characteristics of the parent breeds were transmitted to and expressed in their hybrid daughters. The high incidence in the first generation suggests that these factors were complimentary in action, and the increased susceptibility of hybrid groups derived from the more susceptible breeds, together with the relatively low incidence in animals obtained from the crossing of less susceptible breeds is contributory evidence to this effect. The lowered incidence in second and backcross generations, on the other hand, indicates the expected reassortment of characters with an approach to the status of pure breeds.

Constitutional Variation

In previous paragraphs, emphasis has been placed on the marked differences in susceptibility noted in various pure bred and hybrid

groups and the question arises as to whether such differences were referable to racial distinctions or to other constitutional factors incorporated in the stock by chance association. The genetic constitution of the colony has been under investigation for a number of years and the majority of the breeds are known to carry detrimental variations. In many instances pure bred and hybrid transmitters and non-transmitters have been isolated by breeding tests and the comparative importance of the genetic variation and of race as factors in the determination of susceptibility may be gauged.

Examination of Table III shows that the highest incidence in all the various groups of pure bred animals and their hybrid derivatives occurred in the Polish in which there were 27 cases of toxemia, or 37.5 per cent of the total incidence. The animals of this group had been bred largely for study of an hereditary variation characterized by a dwarfing effect which in homozygous form is lethal and produces a miniature individual approximately one-third the size of its normal sibs. Heterozygous animals are approximately two-thirds the weight of their normal sibs at birth, never attain an equal stature and are subject to a variety of functional disorders (4).

68 of the 115 pure bred and hybrid Polish had been found by breeding tests to be transmitters of this variation. There were 18 cases of the disorder in this group, an incidence of 26.4 per cent, in contrast to an incidence of 19.1 per cent in non-transmitters of the same derivation, or an incidence of 9.2 per cent in all non-transmitters of the colony.

One line of our Dutch breed was known to transmit a cretinoid abnormality and 71 animals had been bred from this line for study of the variation (5). 14 of these died of the disorder, an incidence of 19.7 per cent, which is significantly different from an incidence of 6.06 per cent in animals derived from other branches of this breed, or of 10.0 per cent in the remainder of the colony.

The cretinoid abnormality is apparently a genetic syndrome which may be split into its various parts by breeding, and different features of the disorder may be transmitted and inherited independently, while the variation is expressed in its typical form only when its various component parts are recombined in an individual. Breeding tests had shown that the typical variation occurred in the litters of 45 of the 71 animals while the young obtained from the remaining 26 showed only suggestive changes or were entirely normal. The disorder occurred in 15.5 per cent of the first group while in the latter group the incidence was 26.9 per cent. In view of the marked difference in incidence it would appear that transmitters of the entire complex were less susceptible than other animals of the class. It should be emphasized in this connection that numerous breeding tests are necessary to determine the genetic status of an individual, and it is possible that animals classified as partial or non-transmitters died before adequate tests had been made. On

the other hand, it may be that the accessory factors present in transmitters of the entire complex give rise to a constitutional change which alters susceptibility. A complete interpretation of this apparent paradox is not possible on a basis of the present knowledge of the genetics of the variation, but the latter view is given support by the fact that the incidence among hybrid transmitters of both the dwarf and cretinoid abnormalities was 12.5 per cent which is significantly less than the incidence among transmitters of the dwarf variation alone.

The influence of the lethal dwarfing factor in the determination of the susceptibility of pure bred and hybrid Polish is evident, but the relatively high incidence in non-dwarf transmitters of Polish extraction shows that other factors unrelated to this variation and associated with breed were also of considerable importance in this respect. It may be significant that the majority of this group were first generation hybrids. In other breed crosses the first generation approaches the larger breed in size, but the reverse obtains in Polish crosses and such animals are of small stature. It is not improbable, therefore, that the Polish, the smallest of all breeds, carries a dominant dwarfing factor which is expressed in the first generation hybrids and, like the lethal dwarf character, tends toward increased susceptibility.

The presence of the factors concerned in the cretinoid abnormality was also associated with increased susceptibility. The majority of our pure bred and hybrid Dutch carried these factors, but in the few lines in which the abnormality did not occur, the incidence of the disorder was less than the general level of the population. It is apparent, therefore, that the high susceptibility of these animals was not primarily a function of breed factors as in the Polish, but on the other hand, was associated with an hereditary variation incorporated in the majority of the stock.

All deaths in Rex hybrids occurred in a line known to transmit a deformity of the foreleg resembling rickets, a cystic lymphatic enlargement and a blood condition resembling von Jaksch's anemia. In addition, the single case in pure bred Belgians and the majority of cases in Belgian hybrids were in a line that transmitted a lethal metabolic disorder.

Studies of the pathogenesis of the dwarf and cretinoid abnormalities are not complete, but the facts at hand indicate that both are of hypophyseal origin. Moreover, there is evidence that the Belgian and Rex variations also arise from primary endocrine abnormalities.

Cases of the disorder also occurred in breeds and groups of animals that showed or transmitted other physical or functional variations, but analysis showed no significant differences from the incidence in non-transmitters or normal animals, and it is assumed that the susceptibility of these groups was a function of racial factors.

Parent-Progeny Relations

A more detailed examination of the incidence of toxemia with particular reference to the parental rather than the racial relationships

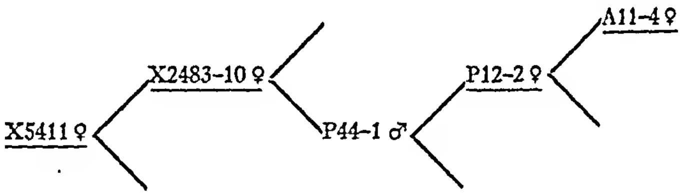
of affected animals brings out additional information regarding the nature of susceptibility.

Analysis of the incidence from this point of view limits the occurrence of the disorder to a relatively small segment of the population, composed in large part of related animals. The total female population of 650 animals had been bred from 140 male and 298 female parents, but the incidence of toxemia was confined to the progeny of 37 of the males and 56 of the females. All cases occurred in animals derived from 62 different matings of these parents, and there were 143 females of this class present in the colony during the outbreak. Thus, on a basis of parental relation the incidence was limited to 22 per cent of the female population of which one-half or 50.3 per cent died of the disorder.

The degree of relationship within this group is indicated by the fact that although the majority of animals were more than four generations removed from the foundation stock of the colony, only 33 males and 39 females of this original stock were concerned in their derivation. It follows that many of the affected

TABLE V

Pedigree Chart Illustrating the Occurrence of Toxemia in Succeeding Generations



Underlined animals died of toxemia.

animals were related through one or both parents. In 5 instances toxemia occurred in full sisters with an incidence ranging from 50 to 100 per cent in the different family groups. 24 animals derived from 9 different females died of the disorder and 45 cases occurred in the progeny of 10 males. In 1 instance 13 affected animals had been derived from a single male and 7 others had been sired by its son.

The disorder was also of frequent occurrence in relatives other than full or half sisters, and pedigree charts of affected animals rarely fail to show additional cases. A simplified pedigree chart illustrating the occurrence of toxemia in succeeding generations is shown in Table V. In 9 instances cases occurred in mothers and daughters during the outbreak while the mothers of 2 other animals had died of toxemia in previous years. In addition, the maternal grandmothers of 9 and the paternal grandmothers of 8 of the affected animals died of the disorder while more remote maternal parents and less immediate female relatives of these and other animals were also frequently affected.

It is clear from the evidence presented above that family as well as race was of considerable importance in the determination of susceptibility. A consideration of immediate parental relations places still greater emphasis on the influence of hereditary predisposing factors.

While classification of the female population into first, second and backcross generations for genetic study is not practicable, some information relative to these factors may be obtained from a differential analysis of the various parent-progeny classes. It should be pointed out, however, that in many instances parents were

TABLE VI

Distribution of Cases of Toxemia Based on Parent-Progeny Relations

	Sons of toxemic doe				Other males				Total	
	Toxemia in progeny (4)		No toxemia in progeny (9)		Toxemia in progeny (40)		No toxemia in progeny (87)			
	No.	Deaths	No.	Deaths	No.	Deaths	No.	Deaths	No.	Deaths
Doe died of toxemia										
Toxemia in progeny (10)	7	1			21	10	8	0	36	11
No toxemia " " (15)	2	0	4	0	19	0	25	0	50	0
Daughter of toxemic doe										
Toxemia in progeny (8)	6	1			17	8	6	0	29	9
No toxemia " " (20)	4	0	4	0	17	0	16	0	41	0
Other females										
Toxemia in progeny (46)	11	8			120	44	20	0	151	52
No toxemia " " (189)	21	0	16	0	136	0	170	0	343	0

Numbers in parentheses represent the number of animals in the various classifications.

not present in the colony during the outbreak period and their status with reference to toxemia was determined at a time when the disorder was sporadic in occurrence while the incidence in the progeny was gauged during an "epidemic" phase. Moreover, many of the affected animals were first generation hybrids and their breeding was designed rather to test their genetic constitution than to supply stock for the colony. As a result, there were relatively few mature progeny present during the period under consideration. In addition it is known that a number of animals of this class as well as of other classes survived the outbreak and have since died with typical clinical and pathological changes. The available data are thus unsuitable in many ways for investigation of the inheritance of susceptibility,

but it is improbable that more adequate material will become available in the future, and as the nature of the disorder prohibits an experimental approach to the problem, the facts at hand are presented in Table VI.

Interpretation of these data is largely conjectural in view of the nature of the material and the small classes formed by detailed analysis. Certain findings, however, are definitely suggestive of the scheme of inheritance. It will be noted that approximately the same incidence of one out of three obtained in the progeny of the three classes of females known to transmit susceptibility regardless of their relationship to the disorder. Moreover, in a number of instances the disorder did not occur in the progeny of daughters of toxemic mothers although these daughters were mated with males known to transmit, and it must, therefore, be assumed that their mothers were not homozygous but were heterozygous for susceptibility factors. It follows that other transmitting females were genetically similar. On the other hand, the fact that cases of toxemia occurred in the progeny of all the sons of toxemic mothers when these sons were mated with known transmitters indicates that some transmitting males may be homozygous. The high incidence in the progeny of certain males previously noted offers contributory evidence to this effect.

Conditions of the Colony in Relation to the Present Outbreak

The high susceptibility of certain genetic groups of animals has been a characteristic feature of this outbreak, but in the past many animals belonging to these groups have been under observation and there have been very few deaths that could be attributed to toxemia. It is apparent, therefore, that the present outbreak cannot be ascribed to the susceptibility of that stock alone. On the other hand, the unusual behavior of the colony as a whole suggests the operation of an extraordinary set of environmental factors which may have been of importance in the genesis of the outbreak.

Disturbances in reproduction were widespread throughout the colony during the outbreak period and began in the spring of 1935. During this season the fertility curve usually reaches a peak and frequently 100 per cent of matings are fertile, but in the spring of 1935 this rise failed to occur and the fertility rate fell below that of the preceding winter. The low fertility continued throughout the outbreak period and the percentage of fertile matings decreased from an average normal rate of 60 to 70 per cent to a rate of approximately 37 per cent. In addition, instances of cannibalism, desertion and other manifestations of poor maternal care were unusually numerous and the incidence of dead born litters and of monsters in these litters was markedly increased.

Other manifestations of disturbed reproductive function were encountered in the colony and are of special significance because of their previous rarity. The

diagnosis of pregnancy by palpation on the 10th day after mating has been found from long experience to be 100 per cent accurate, but during the present outbreak there were cases in which pregnancy had been diagnosed on this date and on reexamination a few days later the product of conception had disappeared from the uterus. This abnormality frequently recurred in subsequent matings of the same animal, and throughout the year there were 52 instances of this kind in 39 animals. It was thought that the resorption of feti might be associated with the deficiency of vitamin E, but the disturbance recurred despite the addition of wheat germ oil to the diet of the animals.

In nddition the incidence of uterine tumor was increased from an average of about three or four a year to approximately 40 during the outbreak period. The tumors were observed in many breeds and hybrid groups, but were particularly frequent in lines of animals in which toxemia occurred. The clinical history and changes found at autopsy in organs of the endocrine system indicate a correlation between irregularity of function and tumor development, and the prevalence of the tumor during the outbreak of toxemia is suggestive of an etiological relationship.

Other manifestations of physiological imbalance were observed in both the male and female population of the colony. The susceptibility to snuffles, a contagious upper respiratory disease in the rabbit, was not markedly altered as gauged by the incidence of nasal discharge. There occurred, however, a change in the manifestations and locus of the disease as shown by the widespread occurrence of abscesses in both internal and external organs which on bacteriological examination showed pure cultures of organisms generally associated with snuffles.

The exact relationship of this series of disturbances and disorders to the outbreak of toxemia is not clear, but it seems likely that some relationship exists, and it is significant that these disturbances were rarities while cases of toxemia were sporadic but occurred in markedly increased numbers when toxemia became epidemic in incidence.

DISCUSSION AND ETIOLOGICAL CONSIDERATION

The similarity of the clinical and pathological manifestations of eclampsia in man and toxemia of pregnancy in the rabbit was noted in a previous paper. Other points of similarity are brought out by a comparison of the incidence of the two disorders and the available evidence indicates that one is a generic variation of the other.

The occurrence of toxemia in both sporadic and outbreak form is a characteristic epidemiological feature. The variations in incidence of eclampsia are apparently not as pronounced, but Williams states that in his experience "it often happens that months elapse without

the occurrence of a single case when suddenly a number are observed in quick succession" (6). Harrar (7) in a study of the occurrence of eclampsia over a 10 year period in the New York Lying-In-Hospital found an increased frequency during the late winter and early spring, and it is significant that the same seasonal distribution occurs in the rabbit, both during sporadic and outbreak periods. It is worthy of note in this connection that a disorder of pregnant guinea pigs, clinically and pathologically identical with toxemia in the rabbit, shows similar variations in incidence (8).

The frequency of eclampsia is greatest in primiparous women while in the rabbit the highest incidence of fatal cases is in multiparae. There is considerable evidence, however, that the first attack of the disorder in the rabbit may be mild or asymptomatic and that death may occur as the result of a repeated attack in a subsequent pregnancy.

The clinics of different countries report varying incidences of eclampsia (7). Lichtenstein reported 400 cases of eclampsia in 14,836 labors in Leipsic, an incidence of 2.68 per cent; Williams found 110 cases in 11,000 labors in Baltimore, an incidence of 1.0 per cent; while Reinburg noted only 90 cases in 26,511 labors in Paris, an incidence of 0.34 per cent. These differences are statistically significant and suggest that racial factors may be of importance in the determination of susceptibility.

The influence of race as a predisposing factor in the rabbit has been noted. Genetic constitution, apart from essential racial characteristics, and family were also found to be of importance in this respect. The scheme of inheritance of susceptibility is uncertain, but is apparently of a complex nature. Females that die of toxemia are apparently heterozygous for susceptibility factors, but there is some evidence that males may be homozygous. Homozygous females presumably never reach maturity but die of unknown causes.

The pathogenesis of eclampsia has not been clarified despite long continued study of human cases, but the disorder in the rabbit has enough in common with it to suggest a common or similar mode of origin. Studies of the etiology of the condition in the rabbit are not sufficiently advanced to allow final conclusions, but certain points in connection with the incidence and manifestations are apparently of significance.

The clinical manifestations and many of the pathological changes as in eclampsia are suggestive of an intoxication, but investigation gave no evidence of a toxic substance of extraneous origin and bacteriological examination was negative. Moreover, the occurrence of typical cases of toxemia in pseudopregnant and in postpartum animals eliminates the possibility of a toxic agent arising from the products of conception.

The incidence of cases, however, leaves no doubt that the disorder is associated with the physiological changes incident to pregnancy and the time of occurrence of cases in relation to gestation is apparently of significance. The great majority of cases in pregnant does appeared during the last few days of gestation, cases in pseudopregnant animals occurred in approximately the same interval after mating, and the majority of postpartum cases appeared on the 1st or 2nd day. The behavior observed repeatedly in pseudopregnant does at this period indicates that these animals are subject to the same physiological changes that characterize the terminal stages of pregnancy, and presumably the influences affecting such changes are still active early in the postpartum period.

During the terminal days of pregnancy fetal growth is retarded (9) and the habitus of the mother is in the process of change in preparation for parturition and lactation. The initiation and control of these processes is a function of the endocrine system, particularly of the hypophysis, and it may be assumed that dysfunction of these glands would lead to abnormal stimulation and pathological alteration of the the processes associated with this period. The marked histological alteration of the endocrine system in toxemia of pregnancy in the rabbit is evidence of dysfunction and is worthy of consideration as a possible factor of primary etiological importance.

The suprarenal changes in toxemia are degenerative in character and probably secondary and the thyroid is hypoplastic and inactive. Hypophyseal changes, on the other hand, are productive in nature. There is marked hyperplasia of the cells of the anterior lobe with evidence of irregular secretion. Alteration of the intermediate lobe is also a constant feature of the disease. The cells of this lobe are markedly increased in number and frequently show adenomatous proliferation or invasion of the anterior or posterior lobes. In addition, there is a large amount of interstitial colloid substance, and cysts

containing similar material are common both in the intermediate lobe and in the posterior lobe where Herring bodies are also unusually distinct and numerous.

There have been recent attempts to associate eclampsia in man with hypophyseal dysfunction, especially with an altered or excessive secretion of the posterior lobe (10), but the relationship is not generally accepted and the findings have not been substantiated (11). However, the increased susceptibility to toxemia of pregnancy among rabbits known to transmit abnormalities of an hypophyseal order, together with the microscopic alteration of the gland in the disease, is indicative of an etiological relationship.

The meaning of the histological changes in the intermediate lobe is not clear, but they are indicative of pronounced secretory activity and may be of significance in the disorder. It should be pointed out in this connection that in normal rabbits the microscopic appearance of the cells of this lobe suggests an active secretory function, and it seems improbable that this function is limited to the production of a chromatophorotropic substance.

There is as yet no satisfactory explanation for the occurrence of the present outbreak of toxemia. Cases of the disease have occurred sporadically in the colony for a number of years, but in the period under discussion the incidence assumed epidemic proportions. There has been no evidence, however, to suggest that the disorder was infectious in character. Bacteriological examination has been negative, cases were distributed irregularly throughout the colony and intimate contact between affected and susceptible animals failed to reproduce any symptoms of the disease.

The character of the population has not changed and many animals of the most susceptible genetic groups were present in the colony during periods of low incidence. There is no indication that the course of the outbreak was related to the changed environment of the colony in its new location, but on the other hand, there is considerable evidence of the continued action of an extraordinary set of influences in both locations.

Examination of the records of the colony shows the occurrence of a widespread disturbance in physiological function in the periods preceding and coincident with the outbreak, and it is noteworthy that

previous to the epidemic of rabbit pox in 1932 these same changes were observed in the animals (2). In addition, there was a profound change in organic constitution during the outbreak period, and autopsies of all animals, both those killed for disposal and those dying of toxemia and other causes, have shown an abnormal endocrine situation. There is normally a constant relationship between the weights of the thyroid and the hypophysis, both increasing and decreasing together in different periods of the year (12). Throughout the period, however, this relationsbip was drastically altered and the hypophysis was extremely large and the thyroid so small that it could be located only with difficulty. It is worthy of note in this connection that in the earlier work of this department when animals were inoculated with syphilis or tumor under similar endocrine conditions, a disease of marked and unusual severity resulted.

It seems probable that there is a causal relationship between the endocrine abnormality and the physiological disturbances observed in the animals, and when considered in association with the hypophyseal alteration characteristic of toxemia, this finding assumes significance as a factor in the genesis of the outbreak. The high susceptibility of animals that transmit and themselves show evidences of hypophyseal abnormalities is also suggestive in this respect, for it may be assumed that in such animals the effect of the changed endocrine relationship would be more pronounced than in the general population.

The nature and mode of action of the influences responsible for the endocrine variation are not known, but the marked effect of various environmental factors on endocrine weights and relationships has been demonstrated (12) and such factors may be the basis of the present imbalance.

SUMMARY AND CONCLUSIONS

No definite conclusions relative to the etiology of toxemia of pregnancy in the rabbit can be drawn from the evidence obtained to date, but certain findings are suggestive and will be investigated further in future studies. These findings suggest that the disorder in the rabbit is a generic variation of eclampsia in man. The incidence, clinical manifestations and pathological lesions indicate that the disorder is of

hypophyseal origin and that the association with pregnancy is due to altered activity of that gland in the terminal stages of gestation.

Hereditary factors related to race and certain constitutional variations were associated with increased susceptibility, but their expression was apparently dependent upon environmental conditions. The association of widespread reproductive disturbances with the outbreak of toxemia suggests a causal relation, and it is assumed that the endocrine imbalance was a primary factor in their genesis and was induced by changed environmental conditions. The general response of the population was manifest in functional disturbances which were of minor severity in normal groups and were expressed as toxemia in inherently susceptible animals.

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THE EFFECT OF FORMALDEHYDE ON PNEUMOCOCCI

By RENÉ J. DUBOS, Ph.D.

(From the Hospital of The Rockefeller Institute for Medical Research)

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Encapsulated pneumococci treated with sufficient concentrations of formaldehyde retain for some time their characteristic morphology, their positive reaction to the Gram stain, and their specific agglutinability in homologous antiserum. Moreover, rabbits and horses immunized by the intravenous route with formolized encapsulated pneumococci react with the production of the specific antibodies directed against the capsular polysaccharide of the bacterial cell used as antigen. It is generally considered, however, that pneumococcus antigens prepared by this technique are not very stable, and undergo with time a form of lysis accompanied by a loss of antigenicity (1).

It has been shown in a previous paper that the "capsular polysaccharide antigen" of Pneumococcus can be rendered ineffective by the action of an autolytic enzyme present in this bacterial species (2). Although this autolytic enzyme can be inactivated by a number of reagents, the inactivation is in many cases reversible (3-6). When, for instance, iodine is used in proper concentration to kill and "fix" pneumococci, the autolytic enzyme is inactivated and the cells retain their structure and their antigenicity; upon removal of the iodine, however, the enzyme may recover its activity and bring about lysis accompanied by loss of antigenicity (2, 5).

In the present study, an attempt has been made to analyze the action of formaldehyde on pneumococci in the light of the observations outlined above. Techniques to render stable the formolized pneumococcus antigens are also described.

EXPERIMENTAL

The bacteriological and immunological methods used in this study are the same as those described in a previous paper (2).

A commercial preparation of formalin (38 per cent formaldehyde) was used as source of formaldehyde.

The Effect of Different Concentrations of Formaldehyde on the Autolysis of Pneumococci.—A great many antiseptics have the apparently conflicting properties of activating the autolysis of different bacterial species when used in low concentrations, and of completely inhibiting

TABLE I

The Effect of Different Concentrations of Formaldehyde on the Viability and Autolysis of Pneumococci

Final concentration of formaldehyde	Growth on blood agar plates			Microscopic appearance of the cells	
	Formolized cultures incubated at 37°C. for				
	3 hrs.	24 hrs.	96 hrs.	24 hrs.	96 hrs.
per cent					
0.5	−	−	−	Gram-positive	Gram-positive
0.2	−	−	−	"	"
0.1	−	−	−	"	Mixture of Gram-positive and Gram-negative
0.05	−	−	−	Mixture of Gram-positive and Gram-negative	Gram-negative detritus
0.03	+	−	−	Gram-negative	" "
0.02	+	−	−	"	" "
0.01	+	+	−	Gram-positive	" "
0.005	+	+	−	"	" "
0	+	+	−	"	" "

\+ indicates growth of pneumococci on blood agar plates.
− " no growth of " " " " "

the autolytic process when used in higher concentration (7, 8). The effect of different concentrations of formaldehyde on cultures of pneumococci is considered in Experiment 1.

Experiment 1.—A plain broth culture of Type III pneumococci 8 hours old was distributed in 5 cc. amounts into test tubes; the culture had reached a final pH of 7.1. Varying amounts of formaldehyde were added to the different samples to give final concentrations ranging from 0.005 to 0.5 per cent, and the formolized cultures were incubated at 37°C. and examined after different intervals of time. The presence of living cells was determined by streaking the cultures on blood agar plates, and the degree of autolysis was followed by microscopic examination of films stained by the Gram technique. The results are presented in Table I.

The results of Experiment 1 show that an amount of formaldehyde corresponding to a final concentration of 0.05 per cent was sufficient to sterilize a culture of Type III pneumococci in 3 hours at 37°C. This amount, however, was not capable of preventing autolysis. In fact the cells treated with the smaller concentrations of formaldehyde (0.02 to 0.05 per cent) underwent autolysis more rapidly than the control cells. The cells treated with the largest concentrations of the antiseptic (0.2 per cent and 0.5 per cent) remained well formed and Gram-positive; in other words they were fixed by the reagent.

Partial Lysis of Formolized Pneumococci Washed Free of Formaldehyde.—The cells in Experiment 1 had remained in the presence of formaldehyde throughout the period of observation; the behavior of pneumococci killed with an amount of formaldehyde sufficient to inhibit the autolytic enzyme, then washed free of the antiseptic, is considered in Experiment 2.

Experiment 2.—500 cc. of plain broth culture of Type III pneumococcus was treated with enough formaldehyde to give a final concentration of 0.5 per cent. The formolized culture was kept at room temperature for 24 hours; the cells were then separated by centrifugalization, washed twice, and resuspended in 50 cc. physiological saline solution. The bacterial suspension consisting of well formed Gram-positive cells was then divided into two equal fractions, one of which received immediately 0.5 per cent formaldehyde. Both fractions were kept at room temperature and the microscopic appearance of the cells followed by the Gram stain.

The cells maintained in the presence of formaldehyde remained well formed and Gram-positive throughout the period of observation (4 weeks). Some of the cells in the suspension washed free of the antiseptic had on the contrary become Gram-negative within 24 hours, and hardly any Gram-positive cocci could be seen after 72 hours. The change from a Gram-positive to a Gram-negative state was accompanied by a reduction in size of the cocci, but no real disintegration of the cells could be observed. No further evidence of lysis appeared on prolonged incubation. The experiment was repeated at ice box temperature (5°C.) and at 37°C. The results were identical with the only difference that the rate of change was slower at 5°C. and faster at 37°C. It is therefore apparent that whereas pneumococci treated with an excess of formaldehyde retain their morphological

and staining characteristics as long as they are kept in the presence of this agent, they may undergo a limited form of lysis when washed free of the antiseptic.

Factors Affecting the Lysis of Formolized Pneumococci.—It has been shown elsewhere that the inactivation of some of the pneumococcus enzymes by iodine is a reversible process; these enzymes recover their activity when the iodine is removed by reducing agents (5, 6). It appeared possible that the lysis suffered by formolized pneumococci washed free of formaldehyde was due to the reactivation of some autolytic enzyme. To test this hypothesis, formolized pneumococci were kept under a variety of experimental conditions in order to control the action of the autolytic enzymes.

Experiment 3.—20 cc. of formalin were added to 1500 cc. of a young culture of Type III Pneumococcus. The formolized culture was kept at room temperature for 24 hours, then divided into six equal fractions and centrifugalized. The formolized cells were suspended in 5 cc. amounts of the following media.

(*a*) M/20 phosphate buffer pH 7.0
(*b*) " " " " " + 0.1 cc. formalin
(*c*) " " " " " ; this suspension was immediately heated at 75°C. for 20 min.
(*d*) " K_2HPO_4 (final reaction pH 8.0)
(*e*) M/20 acetic acid (final reaction pH 4.2)
(*f*) Lugol iodine solution

These cell suspensions were kept at 37°C. and stained by the Gram technique after different intervals of time.

After 24 hours, the cells in suspension (*a*) (neutral reaction, unheated and in the absence of additional formaldehyde or iodine) had become Gram-negative. In all the other preparations, the cells remained Gram-positive throughout the period of observation (2 weeks at 37°C.).

In other words, the change from a Gram-positive to a Gram-negative state is inhibited by the presence of formaldehyde or iodine, by heating the cell suspension, or by maintaining it at an acid or alkaline reaction. These findings may be interpreted as follows. The change from Gram-positive to Gram-negative is caused by an enzyme which is inactive in the presence of formaldehyde (*b*) or iodine (*f*) but which recovers its activity when the formaldehyde is removed and the prepa-

ration is incubated at neutral reaction (*a*); the enzyme is irreversibly inactivated by heating at 75°C. (*c*) and does not function at acid (*e*) or alkaline (*d*) reaction. To substantiate this hypothesis it was of interest to study the effect of the autolytic enzyme of Pneumococcus on formolized cells of this bacterial species.

The Effect of the Autolytic Enzyme of Pneumococcus on Formolized Pneumococci.—It is known that the autolytic enzyme of Pneumococcus can be obtained in an active form from autolysates and extracts of pneumococci (9, 10, 5). The effect of this enzyme preparation on formolized pneumococci is described in the following experiment.

Experiment 4.—3 cc. of formalin were added to 300 cc. of a young culture of Pneumococcus Type III in plain broth. The formolized culture was allowed to stand for 24 hours at room temperature. The cells were then separated by centrifugalization, washed once in saline, and divided into two equal fractions. One fraction was resuspended in 15 cc. M/20 K_2HPO_4 (the final pH of the suspension was 7.8); the other was resuspended in 15 cc. M/20 phosphate buffer at pH 7.0 and this suspension immediately heated at 75°C. for 20 minutes. Both suspensions consisted of well formed Gram-positive cells.

The bacteriolytic enzyme was prepared according to a technique previously described (5). The cells from 150 cc. of a broth culture of R Pneumococcus, derived from Type II, were resuspended in 15 cc. of distilled water and allowed to autolyze in the presence of toluol for 48 hours at 37°C. The Gram-negative detritus was then removed by centrifugalization and the active supernate used as enzyme.

To 1.0 cc. amounts of the two formolized bacterial suspensions (heated at pH 7.0, or resuspended in M/20 K_2HPO_4 and unheated) was added 1.0 cc. to 0.1 cc. of the solution of bacteriolytic enzyme. The mixtures were made up to 2.0 cc. volume with saline and incubated at 37°C. for 24 hours. At the end of this time films of the different mixtures were stained by the Gram technique to estimate the action of the enzyme on the two bacterial suspensions.

It appears from the results of Experiment 4 that the bacteriolytic enzyme of pneumococcus is capable of attacking pneumococci killed with formaldehyde. The enzymatic digestion however does not bring about a disintegration, a real "lysis" of the cell bodies; on the contrary the cells become Gram-negative, but retain their general morphology. In this respect, formolized cells differ from heat-killed cells which are completely disintegrated by sufficient amounts of the bacteriolytic enzyme. It is of special interest that the enzyme renders the formolized cells Gram-negative in a medium at pH 7.8,

i.e. under conditions where the formaldehyde is not likely to dissociate from its combination with the cell structure. Since formolized pneumococci kept at pH 7.8 do not of themselves become Gram-negative, whereas they can be attacked by an active preparation of the enzyme, it seems logical to assume that fixation by formaldehyde is due not to its action on the cell substrate, but to the inactivation of the bacteriolytic enzyme. The results of Experiment 3 indicate that under proper conditions, the fixation of the cell, and therefore the inactivation of the enzyme, are reversible phenomena.

The Effect of Formaldehyde upon the Type Specific Antigenicity of Encapsulated Pneumococci.—Fresh preparations of formolized encapsulated pneumococci (Gram-positive) are capable of inciting the pro-

TABLE II

The Effect of the Bacteriolytic Enzyme of Pneumococcus on Formolized Pneumococci

Amount of enzyme	Appearance of cells at end of incubation period	
	Formolized cells at pH 7.8	Formolized cells heated at pH 7.0
cc.		
1.0	Gram-negative cocci	Gram-negative cocci
0.1	" "	" "
0	Gram-positive cocci	Gram-positive cocci

duction of the type specific carbohydrate antibodies when injected into rabbits by the intravenous route. It has been shown elsewhere however, that the capsular polysaccharide antigen of pneumococci is inactivated by the action of an autolytic enzyme obtained from the same bacterial species (2). It appeared therefore possible that the partial lysis suffered by formolized pneumococci washed free of the antiseptic (Experiments 2 and 3) would be associated with a loss of type specific antigenicity. This is illustrated in the following experiment.

Experiment 5.—The same cell suspensions described in Experiment 3 were used for the immunization of rabbits by the intravenous route. The immunizing dose was the equivalent of 2 cc. of culture daily; three rabbits were used for each preparation. The animals were bled 1 week after the completion of the second course of immunization; their sera were tested for the presence of type specific agglutinins and for precipitins for the homologous capsular polysaccharide (Type III).

The sera of the three animals immunized with the formolized cells which had become Gram-negative (preparation (*a*)) failed to show any type specific agglutinin or precipitin in any dilution. On the contrary the sera of all the animals which had received the formolized cells maintained Gram-positive by destruction or inactivation of the autolytic enzyme exhibited high agglutinating titers and also precipitated the capsular polysaccharide of Type III Pneumococcus. Although there were, of course, marked individual variations between the animals, the best titers were obtained with preparations (*c*) (formolized cells heated at 75°C.) and (*d*) (formolized cells resuspended in dibasic potassium phosphate). Several other experiments were therefore instituted to confirm the antigenic efficacy of these two bacterial suspensions. Twenty-four rabbits were immunized with formolized-heated pneumococci Type III and 18 with formolized cells resuspended in M/20 dibasic potassium phosphate; the amounts of antigen used were the same as in Experiment 5. The sera of the forty-two animals showed type specific agglutinins in titers ranging from 1:40 to 1:640; the prozone phenomenon was observed with the sera of the highest titers. All but four sera were capable of precipitating the capsular polysaccharide of Type III Pneumococcus; the precipitin reaction was very intense in the best sera and was still detectable in a dilution of serum of 1/20. No appreciable difference could be detected between the two antigen preparations.

DISCUSSION

Formaldehyde, when added in sufficiently large concentration to cultures of pneumococci, acts as a fixing agent and preserves the Gram-positive character and the morphological integrity of the cells. When used in lower concentrations on the contrary, it activates markedly the autolytic disintegration of the cells of the same bacterial species. A similar phenomenon has been observed with different types of antiseptics acting on different microorganisms, namely that the same agent may activate, or on the contrary completely stop, the autolytic process according to the concentration in which it is used (7, 8). It has been suggested elsewhere (8) that large concentrations of the antiseptic (formaldehyde in this case) inactivate the autolytic enzymes, whereas smaller concentrations, by interfering with the normal physi-

ology of the cell, cause the autolytic enzymes to act on their specific cellular substrate.

When the cells fixed with sufficient concentrations of formaldehyde are washed free of the antiseptic and resuspended in a physiological medium at neutral reaction, they undergo a partial lysis. The lysis is not complete however, but leaves the cells in the form of small Gram-negative cocci; this "first phase of autolysis" had already been recognized by the use of other techniques in an earlier study (5).

What is the nature of the reaction which renders the formolized cells Gram-negative? One might assume that formaldehyde combines in a reversible manner with the substance or structure responsible for the Gram stain, and that the Gram-positive property is lost when the formaldehyde is allowed to dissociate from its complex with the cell. The following facts are in agreement with this hypothesis. Formaldehyde dissociates from its combinations more readily at pH 7.0 than at pH 8.0, and it has been shown in Experiment 3 that formolized cells may become Gram-negative at pH 7.0 but remain Gram-positive at pH 8.0. On the other hand however, formolized cells resuspended in acetic acid (pH 4.4) also remain Gram-positive although the formaldehyde complexes are least stable at acid reactions. It appears therefore that mere dissociation of formaldehyde from the Gram-positive structure cannot account for the change in staining reaction. The following theory seems to provide a satisfactory explanation of the facts described in Experiment 3.

Pneumococci are known to contain an enzyme capable of destroying the Gram-positive structure of the cells of this bacterial species (9, 10, 5). This enzyme is inactivated by a number of reagents, for example, iodine and formaldehyde. It has been shown that iodine inactivation is reversible (3, 4). We may suppose that formaldehyde inactivation also reverses when the bacterial cells are washed free of the antiseptic at neutral reaction; under these conditions, the enzyme recovers its activity and renders the cells Gram-negative. When, however, the formolized cells are rapidly heated at 75°C., the enzymes are destroyed and no autolysis can take place even at pH 7.0.

Both acid (pH 4.2) and alkaline (pH 7.8) reactions inhibit the action of the enzyme but through an entirely different mechanism. In the acid medium, the enzyme is freed from its combination with formalde-

hyde; but pH 4.2 is outside the range of enzymatic activity and therefore no lysis can take place. On the contrary it has been shown in Experiment 4 that an active preparation of the enzyme can attack formolized cells at pH 7.8. Since formolized pneumococci do not undergo any autolytic change when kept at this reaction, we are led to assume that the enzyme is maintained in an inactive form in the alkaline medium.

On the basis of these observations it is possible to prepare stable suspensions of formolized pneumococci by the following method. Enough formaldehyde is added to the culture to give a final concentration of 0.5 per cent; this stops immediately all autolytic process. The cells can then be separated at leisure from the medium and resuspended under such conditions that no further autolytic action can take place, for instance in a solution of M/20 dibasic phosphate or in an acetate buffer at acid reaction (pH 4.2), or in any physiological solution in which they are immediately heated at 75°C. for 20 minutes.

Cells of encapsulated pneumococci treated by any of these methods have proven to function as very effective type specific antigens even when used in small amounts; they incite rabbits to produce high titers of the type specific antibodies directed against the capsular polysaccharide of the bacterial cells. The results obtained with Type III Pneumococcus are the more striking when one considers that cells of this particular type are notoriously poor antigens. On the contrary, formolized pneumococci which have been allowed to become Gram-negative by removal of the antiseptic and incubation at neutral reaction, entirely fail to incite the production of the type specific carbohydrate antibodies in rabbits immunized by the intravenous route. This finding once more emphasizes the close correlation between the particular antigen concerned, and the structure responsible for the Gram-positive character of the cell (2).

The experimental results described in this paper deal with the effect of formaldehyde on Type III Pneumococcus. Identical results, however, have been obtained with pneumococci of Types I and II, and the methods of preparation of pneumococcus antigens outlined above have been used successfully for the production in rabbits of therapeutic antisera for the different pneumococcus types (11).

SUMMARY

When used in low concentration, formaldehyde increases the rate of autolytic disintegration of pneumococci whereas in large concentrations it completely inhibits autolysis and preserves both the morphological and staining characteristics of the cells.

Pneumococci treated with large concentrations of formaldehyde, then washed free of the antiseptic and resuspended in physiological solutions, rapidly undergo a change which renders them Gram-negative and smaller. The lysis is only partial, however, and is not accompanied by an actual disintegration of the cell. It is caused by the autolytic enzyme of the cell which remains inactive in the presence of an excess of formaldehyde but recovers its activity when the cells are resuspended in a neutral medium after removal of the antiseptic. If the autolytic enzyme is irreversibly inactivated by heating, or maintained inactive in acid or alkaline reaction, the formolized cells retain their staining characteristics and morphological integrity.

Formolized pneumococci which have become Gram-negative owing to the action of their autolytic enzyme, fail to elicit the type specific carbohydrate antibodies in rabbits. Formolized pneumococci in which the autolytic enzyme has been destroyed or maintained inactive, and which have retained their Gram-positive character, function as a very effective type specific antigen in the rabbit.

These observations emphasize once more the close relation between the Gram-positive structure of pneumococci and the capsular polysaccharide antigen of the cell. They can be used as a basis for the preparation of suspensions of formolized pneumococci which are stable and very effective as type specific antigens.

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THE CARCINOGENIC EFFECT OF A PAPILLOMA VIRUS ON THE TARRED SKIN OF RABBITS*

I. Description of the Phenomenon

By PEYTON ROUS, M.D., and JOHN G. KIDD, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

Plates 13 to 18

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Virus diseases involve reciprocal activities on the part of the infected cells, no matter how soon these die, and their opportunity to influence the situation becomes greater the longer they endure. When they proliferate in series, the resulting growths must be looked upon as the expression of a working partnership with the virus, a state of affairs evident in various tumors of the domestic fowl, and in the infectious papillomas of several mammalian species. Ordinarily these growths, as propagated in the laboratory, are the outcome of infection of the cells of an acutely damaged tissue; and all those caused by the action of any one virus are essentially alike. In the present paper and others to follow it will be shown that tumors of widely differing character will result from the action of a single virus if the cells are appropriately altered before it comes into association with them.

The virus employed for our experiments was that which causes the cutaneous papillomas of western cottontail rabbits (1). On inoculation into domestic rabbits it gives rise to similar growths which often undergo malignant change, carcinomas arising directly from their epithelium (2). This change has never occurred until after several months, in our experience; but various interferences which enhance the cellular proliferation and render it disorderly act to shorten the precancerous period, and they may elicit cancer forthwith when the papilloma has been growing for a long time. Such occurrences have led us to ask whether cancer will develop at once if the virus is brought

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into association with epidermal cells that are already in a pathological state. This is the case, as will be demonstrated.

Rationale

Many agents causing epithelial disturbance and proliferation fail to bring about the tissue alterations preliminary to cancer. For our purpose some substance was needed that would prepare the cells suitably, yet that could be utilized without likelihood that it would itself elicit cancers under the conditions of the experiments. Such a substance was available, namely a tar with which we had done much work, the horizontal retort tar of the Oster-Gasfabrik of Amsterdam.[1] It elicits cancer within a few months in mice (3), and benign "warts" in domestic rabbits (4); yet cancer appears in these latter only after a very long time and then rarely. Our rabbits were tarred on the ears until a few small warts had appeared on some of them, and then were inoculated with virus. Indications had already been obtained of slight differences in the outcome of individual cell-virus associations, as expressed in the growths consequent upon them (5), and hence it seemed important to scatter the virus entities to individual cells altered in varying degree by the tarring. With this in view the virus was injected intravenously. It localized in the tarred skin and here promptly elicited carcinomas as well as a variety of papillomas.

Local Effects of the Tarring

The effects of tarring have been often described, yet some account of them as observed in our rabbits is essential. The animals were all agouti (brown-gray) adults weighing about 2500 gm. More than 30 had been utilized previously in an attempt to obtain tar cancers for serological purposes (4), but only one such growth had been got, and this after nearly 2 years, although the tarred skin in most cases underwent the well known changes preliminary to malignancy, numerous "warts" arising, that is to say papillomas and "carcinoids" (6),—growths which look like cancers both in the gross and microscopically, but which remain local, fail to grow on implantation elsewhere in the host (7), and disappear or become mere papillomas if tarring is left off. The experience of others with other tars gives every reason for the supposition that cancer would eventually have arisen in some instances had not nearly all of the animals died of tar intoxication after 6 to 15 months.

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[1] The gift of Dr. Karl Landsteiner.

In the tarred controls of the present work no cancers ever arose. They bring the total number of control animals to more than 90.

The inner surface of the ears was tarred twice a week. In most instances they soon became thickened, warm, and hyperkeratotic, scurfy or macerated; and after 2 to 4 months a few small warts appeared in more than half of the animals. The diffuse histological changes were those often recorded (8), and the growths were the familiar tar papillomas and carcinoids. Not infrequently tumors of the one kind graded into the other.

The later course of events varied widely. The ears of some rabbits remained free from warts, while those of others at the opposite extreme developed numerous growths which, rapidly enlarging, filled the aural shells. Some of the large tumors were predominantly epithelial, and took the form of cauliflowers, cones, or cutaneous horns, but the generality owed their size to proliferation of the connective tissue supporting a thickened, papillomatous epidermis. They often rounded out into tangential spheres, which, as time passed, became pedunculated and underwent retrogression to fibrous tags. Both the papillomas and carcinoids sometimes invaded the connective tissue soon after their appearance, and, extending through lacunae in the cartilaginous plate, formed mounds on the outside of the ear, which occasionally ulcerated; but later they ceased to enlarge and either disappeared or became indolent cones or horns. Often, though growing swiftly at first, they ran a brief course, disappearing despite the continued application of tar. Leroux (9) has well described the retrogression of such tumors.

The rabbits stripped the ears between the paws to remove the tar, and in this way transferred some of it to the outer side, with result that here the skin lost its hair, became hyperkeratotic, and occasionally developed papillomas. Their rarity in this situation during the first 4 months of tarring deserves stress because the introduction of the virus into the blood stream during this period was often followed by the appearance of hosts of papillomas on the outsides of the ears.

In most of the experiments the virus was injected after tarring had been done for 2 to 4 months, and it was kept up for a few later weeks, though this was not essential to the carcinogenesis, as recent findings have shown. When it was finally discontinued the skin of the ears of most of the control rabbits, which had often been macerated, thickened, and furry, rapidly dried down, and a more or less pronounced desquamation took place, revealing at length a smooth, normal-looking surface. Most of the growths dried down too, and some came away; but others, after persisting for a few weeks as mere scabs, began to grow again, becoming horns or dry cauliflowers, or fleshy cones or onion-shaped masses; and sometimes one or more new ones appeared. When they had been large, crowded, and macerating, as rarely happened, they tended to keep on growing, aided by the maceration, and occasionally reached a diameter of several centimeters in the absence of any further tarring. But these large growths were pedunculated, fibrous, and wholly benign.

Very important for the interpretation of the findings after virus injection was the lack of pronounced pigmentation of the tar tumors, and the situation of nearly all on the inside (tarred side) of the ears. In agouti rabbits an occasional small, indolent tar wart is light or medium gray, or very rarely dark, owing to included melanoblasts; but it regularly loses this color in proportion as it proliferates more actively; and vigorously growing tar tumors are never gray but creamy, buff, or pink. Many of the papillomas induced in tarred skin by the virus were by contrast slaty brown, or coal black; and one could be sure that the virus had a hand in any deeply pigmented, yet actively enlarging papilloma which appeared after its injection. This is not to say that virus was absent from such pink, buff, or creamy growths as also appeared, for it elicited many. Furthermore some virus-induced growths that were at first deeply melanotic often became pink later, as happens with such growths on scarified normal skin (2). The appearance during the 3rd to the 5th week after virus injection of numerous papillomas on the outsides of the ears, many of them slaty, was proof positive of the action of the virus.

Our tar was of moderate "carcinogenic" potentialities, to judge from its ability to elicit papillomas. The one cancer it induced, a squamous cell carcinoma with some papillomatous features, appeared after 21 months in an animal tarred for two periods of 5 and 6½ months. When it was killed, after 656 days, a cystic metastasis with the same papillomatous features was present in an auricular gland.

The aim of the tarring after injection of the virus was to prevent reversion of the epidermal cells to the normal state before it had had time to take effect. In skin directly inoculated with a potent virus material 10 days to 3 weeks ordinarily elapses before the first roughening preliminary to papillomatosis can be noted in the gross; but when the individual virus entities are scattered to the tarred epidermis by way of the blood, the growths often appear later and sometimes not until 2 months or more have gone by.

The Virus Materials and Their Effects

Most of the virus materials were generously given us by Dr. Shope, as glycerolated papilloma tissue from cottontails. Attempts to maintain by passage the pathogenicity of active strains of virus from "natural" growths are not very successful, an inoculum of diminished potency being usually obtained from cottontails, while from domestic rabbits the virus is either not recovered at all or in greatly attenuated form. Large amounts of material of high titer were essential to the work, both because of the dilution inevitable to dispersion of the virus on the blood, and because weak strains of it have little carcinogenic capacity, malignancy seldom supervening upon the papillomatosis that they induce (5). The material principally employed consisted of the natural growths from a single cottontail (1211), and it was exceptionally active, as proven by checkerboard titration (4). In the tarred

skin it promptly elicited carcinomas as well as papillomas in a considerable proportion of the injected animals. Another material, nearly as active, was injected in much smaller amount and the virus localizations and cancers were correspondingly fewer. A third material consisted of the pooled papillomas from 7 cottontails experimentally inoculated with a potent virus. It localized in considerable quantity in the ears of the 15 rabbits receiving it, all susceptible and many with tar warts; yet it gave rise only to slowly-growing, ordinary papillomas. This experiment need not be mentioned further.

The Virus Tumors Arising in Scarified Normal Skin

The growths produced by the virus on direct inoculation into the normal skin of agouti rabbits are all papillomas of a single characteristic sort (1,10), some pink but many gray owing to included and stimulated melanoblasts, elements not rendered neoplastic, however. The individual growths take the form of high cones or fleshy onions, more rarely of cutaneous horns or cauliflowers, and their keratin builds high in dry, vertically ribbed or striated layers. In the gross the papillomas resemble some of the tar tumors (11), notably those which continue to enlarge after tarring has been stopped; but as a group they proliferate much more vigorously and are often distinguishable by a pronounced melanosis, a fact already brought out. The cancers arising from them in the ordinary course of events are never pigmented though. They are often multiple, and range in morphology from complicated papillomas of slight aggressive power to the most anaplastic of metastasizing, squamous cell carcinomas.

General Method

In most of the experiments the tar was applied to the central two-thirds of the inner surface of the ears, whence it spread to the edges. Before every third application as much of the old layer was stripped off as possible. A day or two prior to the virus injection all tar was removed, the warts were drawn to size on standard forms, note taken of the general state of the ears, and on the basis of the findings the animals were separated into comparable groups, one serving as control. The charting was frequently repeated later and all significant changes were noted. In most instances tarring was resumed for 14 to 30 days after virus injection, and then the layer was permanently removed and immediate charting done. The controls were similarly treated save that they received no virus and were kept isolated.

The virus suspensions were made by grinding the glycerolated papilloma tissue with sand, suspending in Tyrode (pH 7.0–7.4), centrifuging briefly, and passing the supernatant fluid through a Berkefeld filter. The filtrate was slowly injected into a vein on the outer side of the thigh.

When biopsy specimens were required from the ears, sharp cork-borers were employed and a blow of the mallet.

THE EXPERIMENTS

For the first experiment three groups of tarred rabbits were employed. Into one a virus filtrate was injected, another was set aside for control, while the third received an incubated mixture of virus suspension and heated cancer extract. This was used because Berry has shown that rabbit fibroma virus incubated with heated rabbit myxoma tissue gives rise to disease of the latter type (12). Ours was an attempt, collateral to the main experiment, to convert the papilloma virus into a carcinoma virus. To learn the effect of tarring on ordinary epidermal cells infected with the virus materials, the latter were tattooed into the insides of the ears of several normal rabbits. Tarring was then begun for the first time.

Experiment 1.—The virus-containing fluid was a 4 per cent Tyrode extract of the papillomas from W.R. 1211, which had been passed through one or another of 3 Berkefeld filters, V or N, and been pooled. The cancer extract was made by grinding with sand and Tyrode the tissue of several large, squamous cell carcinomas that had arisen from virus papillomas. The malignant tissue had been frozen and dried some months previously, after separation from all gross remains of the papillomas. A 7 per cent extract of it by dry weight was heated in a water bath at 65°C. for 30 minutes, centrifuged to throw down gross particles, and the murky, supernatant fluid was mixed with an equal portion of virus fluid and incubated at 37°C. for 3 hours prior to injection, while another portion of the virus fluid, mixed with the same amount of Tyrode, was similarly incubated.

Five rabbits were injected intravenously with virus mixed with Tyrode, 4 with the mixture with cancer extract, while 5 more were kept as controls. All had been tarred over the entire inner surface of the ears during periods of from 42 to 89 days, with result in small warts. On the day after the injections, tarring was resumed and repeated twice weekly during the next 25 days.

Two of the rabbits with more numerous and larger warts than any of the others had been placed in the *control group* (Chart 1). The warts of one dwindled during the later tarring but those of the other (rabbit 6) enlarged even after it had been stopped, and new ones appeared. Maceration furthered their course. When this rabbit was killed after 9 months, its ears bore crowded masses of pedunculated, fleshy papillomas, with a few cutaneous horns, all benign. This instance has proved unique in our experience. Little change occurred in the ears of the other controls during the later tarring, and thereafter most of their growths disappeared. In 2 of them a subepidermal mound had formed on the outside of the ear, opposite an active growth on the inside, as if by extension from the latter, but it dwindled and vanished together with this after tarring had been left off.

In a *subsidiary control test* the two virus mixtures were tattooed, with an electric machine, into 2 strips about 5 mm. wide, extending nearly the entire length of the inside of the ears of 5 normal rabbits. The virus with Tyrode was introduced into the left ear, the mixture with cancer extract into the right, and tarring was then begun for the first time and kept up twice weekly until death. Semiconfluent and confluent papillomatosis developed along all the tattooed strips after about 18 days, and the growths enlarged with a rapidity unprecedented in the case of untarred ears (Chart 3). At first predominantly gray, as when no tarring is done, they soon became high, pink, macerating, cauliflower masses. Their foul state led to early death in 3 cases. All of the growths remained ordinary, virus-induced papillomas throughout the 55 to 84 days of tarring, though sections showed their epithelium to have extended down into the profuse reactive tissue, forming cysts, as often happens in the case of vigorous virus papillomas induced in scarified normal skin (2, 5). Elsewhere on the ears the tarring caused the usual hyperplastic thickening, but no warts. In one animal (No. 2), 2 discrete papillomas appeared off the line of tattoo inoculation, but their slaty color marked them as due to the virus.

From Chart 3, of the growths due to *tattoo inoculations* into normal ears which were tarred later, it will be seen that the incubation with cancer extract had greatly cut down the pathogenicity of the virus. It led to no qualitative difference, though, in the growths engendered, all remaining papillomas of the characteristic sort despite great stimulation by the tar.

The course of events in the *animals injected with virus incubated with Tyrode* (Chart 2) differed notably from that in the controls (Chart 1).

During the 3rd week after the injection many new, rapidly enlarging warts appeared, and most of the old ones began to grow at an unprecedented rate (*vide* the records of the 22nd day). In addition, a diffuse change took place in the ears of rabbits 11, 12, and 13. During the 3rd week after the injection they suddenly became much swollen, stiffened, brawny, and hot, and within the next few days papillomatosis appeared over large areas on both their outer and inner surfaces. It took the form of gray and pink, rugose expanses, or of multitudes of minute growths, mostly gray. Some larger, discrete, rapidly growing tumors appeared as well, and many of the preexisting tar warts suddenly began to grow with unprecedented rapidity. The discontinuance of tarring resulted in no slowing of the proliferation, and the ears were soon almost entirely occupied by growths, some of them malignant in behavior. Only the large tumors were charted: there were too many small ones. The time of the first changes after virus injection corresponded roughly with that when papillomas became noticeable in the tattooed

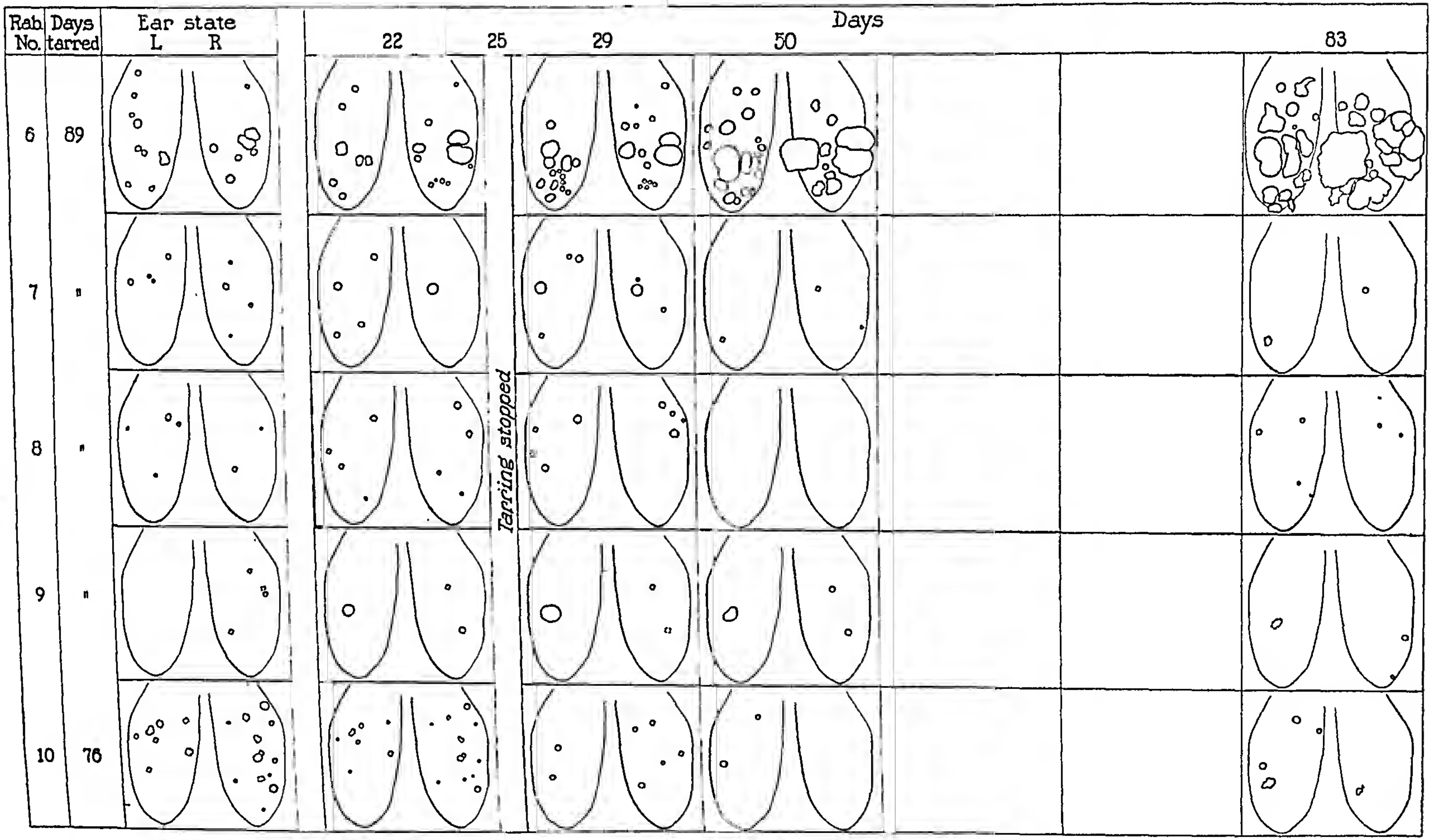

CHART 1. Tarred controls.

CHARTS 1 to 4. Experiment 1. Growths on the inner surface of the ears, before and after virus injection. Minute ones are not charted. Hatching shows areas of confluent proliferation. Along the middle of the ears of rabbits 12 and 13 maceration kept it low, and no charting has been attempted here. Growths of carcinomatous morphology, as determined by biopsy specimens or at autopsy, are shown in solid black from the time when their character was first demonstrated microscopically.

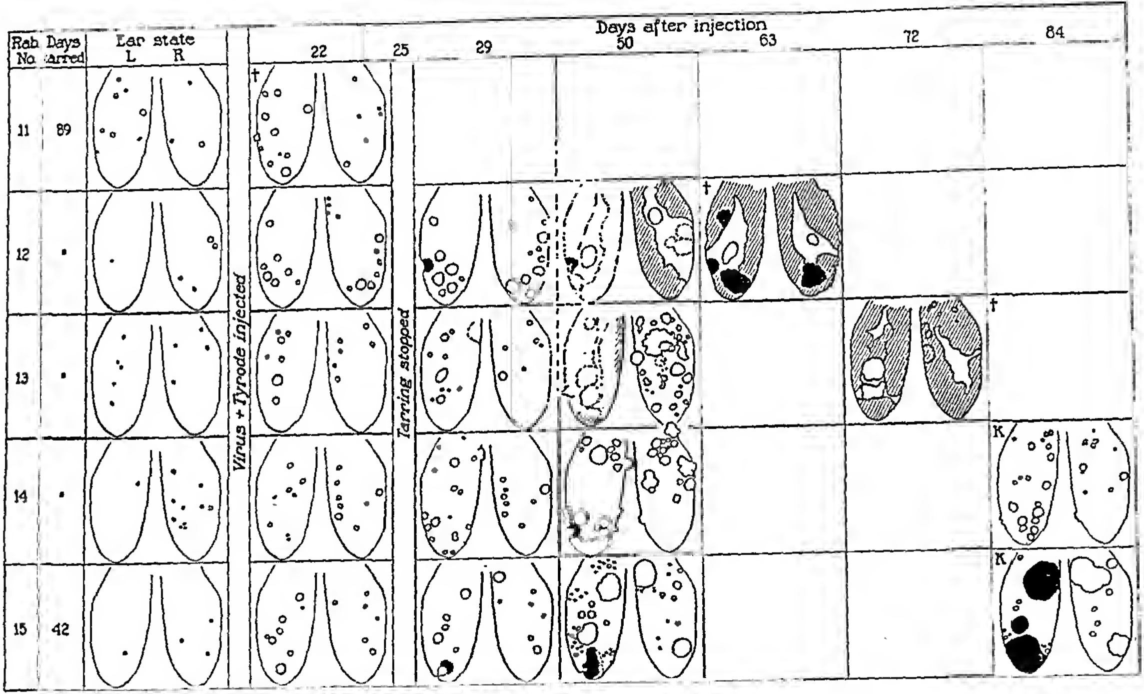

CHART 2. Virus + Tyrode.

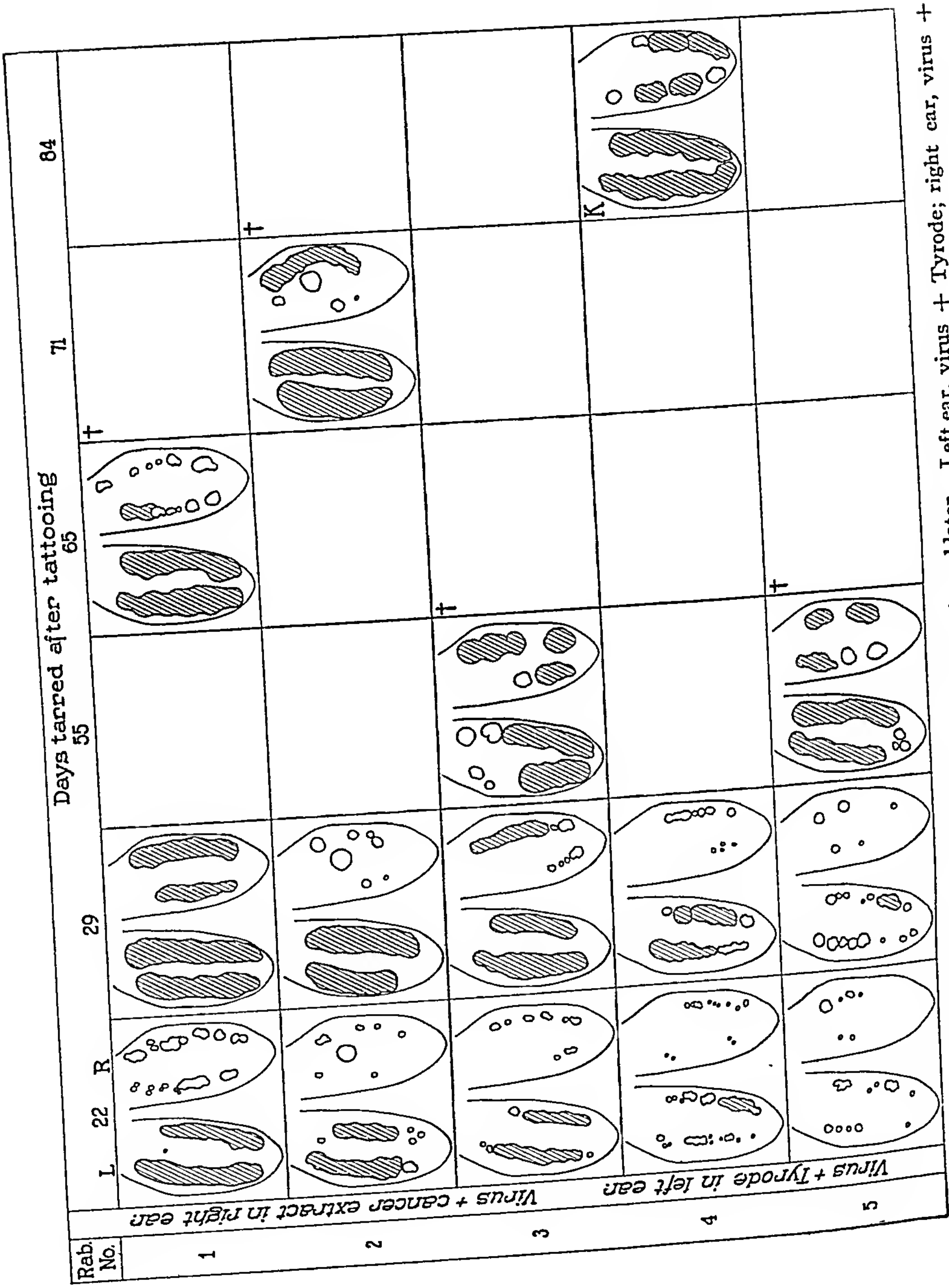

CHART 3. Tattoo inoculation into normal ears that were tarred later. Left ear, virus + Tyrode; right ear, virus + cancer extract.

group. The charts made between the 29th and 50th days have not been reproduced.

Rabbit 11 had thickened, hyperkeratotic ears at time of injection. It died soon after, of intercurrent causes, yet provided much informative material. On the 18th day the ears had suddenly become greatly swollen and brawny; and 2 small, gray, subepidermal mounds had appeared on their outer surface. The swelling increased, more growths appeared, the old grew rapidly (Chart 2), and at death on the 22nd day the ears were from 5 to 10 mm. thick, covered inside with a furry, macerating sheet of fungoid tissue amidst which the old tar warts and some new ones stood forth as slightly higher, discoid growths. Only the latter are indicated in the chart. On section the sheet varied in thickness from 1.5 mm. near the tip of the ear to 7 mm. toward the base, was vertically striated, creamy, streaked and spotted with gray, and consisted microscopically of confluent papillomatous tissue, as if from broadcast, epidermal infection with the virus. The embedded warts, old and new, were also of papillomatous character. Some appeared to be breaking up into squamous cell carcinomas along their base, a frequent finding in ordinary tar warts. Blocks taken at random disclosed occasional localized downgrowths of carcinomatous morphology where no tar warts had existed prior to the injection (Fig. 1).

The skin of the outer side of the ears was irregularly raised, and a cut disclosed numerous separate, discrete, gray or creamy, subepidermal, acorn or onion-shaped growths from 0.5 to 1.5 mm. in diameter. The microscope showed these to be discrete papillomas, deriving from the epithelium of hair follicles and not yet erupted. Some were dark gray.

The early changes in rabbits 12 and 13 were of like sort. The sheets of new tissue which formed on the insides of the ears during the 3rd week after injection soon thickened to 1.0 to 1.5 cm., enveloping the warts previously present or newly appeared, and filling the ears with a high, foul, scabbed mass, save along a central strip where maceration and pressure necrosis kept the tissue low. (Fig. 16 of Experiment 2, illustrates this state of affairs.) Scattered, gray, subepidermal mounds appeared on the outer surface during the 3rd week; and rapidly increasing in size and number these broke through the stretched epidermis, in the case of rabbit 13, and became vigorous, conical or onion-shaped growths with fleshy, bulging, gray bases and dry, sooty, vertically striated peaks,—characteristic virus-induced papillomas in short. Often they coalesced. They were most numerous where the skin was hyperkeratotic and hairless. On the back of the neck, where transferred tar had caused similar, but slighter, skin changes, many discrete or semiconfluent papillomas also arose.

On the 78th day after injection *rabbit 13* died of sepsis. Ruddy fungoid growths up to 2.5 cm. in diameter, some deriving from tar warts, were then present on the inner side of the ears, amidst a thick sheet of foul, vertically striated pink and gray tissue. The microscope disclosed only non-malignant papillomatosis, though the papillomas were of highly various character. Their histology will be

considered in a succeeding paper. Many small abscesses were present in the auricular glands.

The events in *rabbit 12* warrant more detailed description. The ears were slightly thickened and mildly hyperkeratotic at time of injection, and they bore 3 small warts and 2 dubious rugosities. These underwent little change until the 3rd week when some had become raised, granulating discs, while other similar discs had appeared and also ruddy, subepidermal mounds, with scattered gray ones on the outsides. On the 25th day, that of the final tarring, the ears had recently undergone a turgid, hot stiffening. Many new, gray, subepidermal mounds were now present on their outsides, and 4 larger, pink ones had developed there opposite 4 raw, ruddy discs on the inner surface, as if by extension from these. 3 of the inner discs had first become noticeable between the 18th and 22nd days, while the fourth had been present at time of injection, as a small tar wart. Other discrete, ruddy discs or hassocks were also to be noted now on the insides of the ears. On the 29th day the pink, outer mounds had enlarged greatly and 2 were ulcerating. The corresponding discs lay amidst a thick sheet of new tissue covering most of the inner surface of the ear, which sheet is not indicated on the chart. A piece was punched from the disc and mound nearest to the ear margin. It showed what appeared to be an ulcerated, anaplastic, squamous cell carcinoma (Fig. 2), which had extended beneath the adjacent skin, through the ear cartilage, and under a nearby papilloma. During the later weeks, until death on the 63rd day, this growth enlarged but little. The other ruddy, discrete tumors continued to grow however, though obscured by the thick sheet of proliferating tissue covered with heavy, brown scab that rose about them. Under this latter some of them extended widely (Chart 2), while the pink mounds on the outside opposite certain of them became deeply ulcerated. Only the large scabs and the growths discernible through it could be recorded at the late chartings. The numerous, gray, subepidermal mounds on the outside of the ears rapidly enlarged (Figs. 26 and 27) and fused into irregular plateaus covered with breast-shaped, subepidermal protrusions (Fig. 3), each with a nipple-like, dry, black cone at its top. Later sections showed the plateaus to consist of a multitude of keratinizing papillomas of virus type.

On the 50th day a firm, spherical nodule 4 mm. across was felt in a lymph gland at the base of the left ear, the one which carried the biopsied growth. 4 similar growths, with irregular, raised ulcerations opposite them on the outer side, had enlarged progressively; and near the tip of the left ear 2 of these had fused into a thick, fungoid mass.

The animal was sacrificed when moribund, on the 63rd day. The fused growths just mentioned had eaten a foul, transverse furrow, and the ear tip hung limp, attached only by cartilage. The gland nodule had reached 8 mm. in diameter.

Microscopically the 5 aggressive, destructive growths were carcinomas, some anaplastic, others with papillomatous features (Fig. 4). Their extension through lacunae in the cartilaginous plate accounted for the ulcerating mounds on the outer

side of the ears. The numerous other, more or less discrete growths lying amidst the inner masses of fungating, scabbed tissue proved to be papillomas of highly various sorts, none certainly malignant. The greater part of these masses consisted of confluent papillomatosis of ordinary virus type, some of it gray; and the mammillated plateau on the outer side consisted entirely of growths of this kind. Sections from many places were searched for malignant growths that were just arising, but none was found.

The nodule in the basal gland had the morphology of an actively invading squamous cell carcinoma, cystic and with papillomatous features (Figs. 5 and 19). No other secondary growths were found.

The remaining 2 rabbits of the group exemplify the effects of a less abundant localization of the virus in the ears.

Rabbit 15, tarred 42 days when injected, had ears much changed, carrying 3 minute warts. In the 3rd week the growths suddenly increased in number and size. No diffuse thickening or confluent papillomatosis developed. Several of the newly appeared growths were raised, raw discs with depressed centers; and by the 22nd day a pink, subepidermal mound had formed on the outside of the left ear opposite one of them, first noted 4 days previously. On the 29th day, when the mound had ulcerated, a punch biopsy was done, and the growth was found to be a squamous cell carcinoma histologically, which had extended through the cartilage. Later it enlarged rapidly into a broad, fungoid, weeping, lenticular mass on the inner surface of the ear, and a raised ulceration without. So also did 2 similar, discoid growths dating from the 22nd day (Figs. 8, 12). The successive pictures (Figs. 6 to 13) show how quick were the changes. 2 fimbriated, pink growths on the right ear, one of them derived from a preexisting tar wart, also enlarged (Figs. 6, 10). The other, which was new, extended through the cartilage to form a big mound that remained subcutaneous (Fig. 11). In the gross these were mere papillomas. A scattering of discrete, small, sooty or pink growths of the same sort also appeared, notably on the outside of the left ear.

After the 50th day a progressive dwindling took place of many of the smaller growths just mentioned (Chart 2). Few were left by the 84th day, when the rabbit was killed, though the 5 large tumors already described had continued to grow, those of the left ear destructively. The skin between the growths had long since become to all appearance normal. The microscope showed the 3 destructive growths to be squamous cell carcinomas in histology, whereas the 2 on the other ear, though aggressive and somewhat disorderly, were benign papillomas. Many round cells and makrophages were present under and about the small, retrogressing growths, and dark gray spots due to phagocytes crammed with melanin marked where some had disappeared that had been dark gray. There were no metastases.

The ears of *rabbit 14* had been little changed by the tarring. The early events after inoculation were like those in rabbit 15, and soon after tarring was stopped a pink growth appeared which rapidly extended through the ear. Biopsy on the 32nd day showed the growth to be histologically a squamous cell carcinoma. Un-

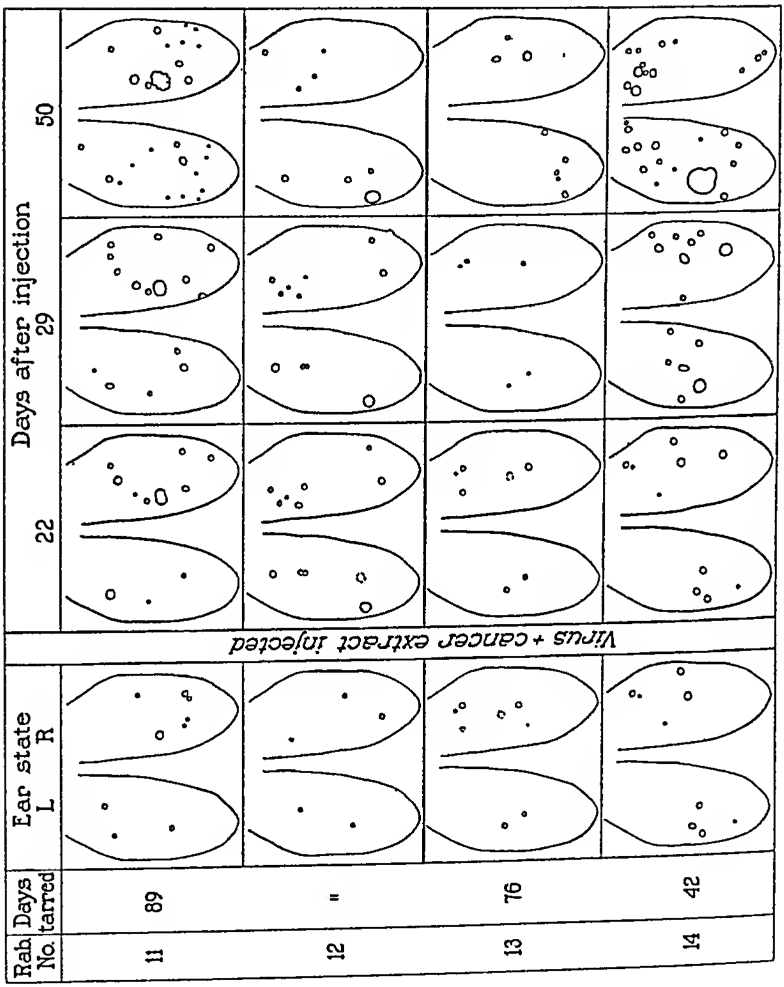

CHART 4. Virus + cancer extract.

fortunately nearly all of it had been taken, and local purulence destroyed the rest. Later some of the fairly numerous growths disappeared, and most of those that persisted to the 84th day, when the animal was killed, were in some degree pedunculated. All were papillomas, and nearly all were dark gray.

In these rabbits, the injection of *virus incubated with Tyrode* was followed by remarkable changes in the ears. After a few weeks, papillomas developed, often in immense number, and in 4 of the 5 animals destructive growths also appeared that behaved like carcinomas and had the morphology of such at early biopsy. They rapidly extended through the cartilage, frequently penetrated into the blood and lymph vessels, and continued to proliferate after tarring had been stopped and even while papillomas on the same ears were retrogressing (rabbit 15). In one animal a secondary growth developed in a lymph node.

The changes were almost negligible by comparison in the animals which received *virus incubated with heated cancer extract.*

In the 3rd week (Chart 4) a few papillomas appeared that were obviously due to the virus, being dark gray, vigorous, rapidly growing, and situated in some instances on the outside of the ears. Others also developed of which there could be no certainty as to cause, since they were creamy or pink. No malignant tumors had arisen by the end of the experiment (84th day), nor did the existing growths alter significantly after the 50th day. Hence the later chartings are omitted.

These findings confirmed the outcome of the tattoo inoculations in showing that the incubation with tumor extract had greatly lessened the pathogenicity of the virus. Indeed the injected animals served as additional controls, testifying to the absence of cancers consequent on the tarring as such.

Sufficient of the virus material of Experiment 1 was available for several more tests. In one the effect was noted of incubation with a cancer extract devoid of inhibitory effect on the virus. The rabbits came from the same batch as those tarred for Experiment 1.

Experiment 2.—A Berkefeld filtrate was made of a 5 per cent extract of material W.R. 1211, and part was mixed with twice its bulk of a heated 8½ per cent extract in Tyrode of a squamous cell carcinoma derived from a virus-induced papilloma. The cancerous tissue had been kept frozen for 16 months. It was ground with sand, extracted with Tyrode, spun to remove particles, and the central portion of the murky, supernatant fluid was taken off through a long needle, and heated at

65° for 30 minutes. The mixture with virus was incubated at 37°C. for 3½ hours and then 13 cc. was injected into a leg vein of rabbits 1-31 and 1-35 N.

In a collateral test of the effect of the cancer extract on the virus filtrate, some of the latter, as such and diluted to 1 per cent, 0.2 per cent, and 0.04 per cent, was mixed with twice its bulk of Tyrode, and with heated and unheated cancer extract respectively, after which it was incubated as above and inoculated into checkerboard squares on the skin of 3 normal rabbits. All of the inocula yielded growths, the heated and unheated cancer extracts having no more effect upon the outcome than the Tyrode. After 9 days papillomas began to appear where the mixtures with 5 per cent virus had been inoculated, and after 16 days and 21 days where 0.2 per cent and 0.04 per cent had been introduced. The number of growths varied directly with the virus dilution.

D.R. 1-31 N, tarred 46 days, had 7 warts, 1 to 6 mm. across when injected with the mixture of virus and cancer extract. Tarring was kept up for 30 days more. During the first 2 weeks after the injection the warts enlarged slowly and no new ones appeared; but within the next 2 weeks a great increase in their size and number occurred (Chart 5). Most of the bigger ones, both old and new, became raised, fungating, ruddy, discoid masses, covered with foul secretion which caused maceration. By the 26th day the largest was 2 cm. across and 5 mm. high. The ears did not dry down after tarring was stopped; the tumors continued to enlarge rapidly, more appearing; and on the 38th day an ulcerated mound was present on the outside of the right ear opposite one of the oldest growths, which had been noted as a slight thickening of the skin at the time of virus injection, and had since become a fungating disc with ill defined borders. Biopsy of it showed a growth with the histology of an anaplastic, squamous cell carcinoma (Fig. 20), which had extended through a lacuna in the cartilaginous plate. It soon caused ulceration on the outer side. Opposite two other, similar discs, first noted on the 24th day after the injection, and now with depressed centers, mounds had appeared, one of which ulcerated in the next few days (Fig. 17). In a region about 2 cm. across, toward the base of the ear, a deep thickening (Y) developed on both sides of the cartilage. A few old growths on the inner surface,—tangential, fleshy spheres or pedunculated cauliflowers, such as tarring frequently elicits,—did not alter noticeably; but elsewhere over this surface great numbers of small, rugose mounds and obvious papillomas arose after the 26th day, and on the outer surface a scattering of subepidermal, breast-shaped mounds, mostly gray, the larger with a central, dark, nipple-like protrusion, consisting of keratinized tissue.

Proliferation continued at a rapid pace, the growths on the inner surface becoming confluent and macerating; and more ulcerating mounds appeared on the outside. Biopsy through one of these latter on the 40th day disclosed another anaplastic, squamous cell growth that had originated on the inner aspect of the ear and extended through the cartilage. The animal, by now very thin, was transfused with 51 cc. of citrated blood on the 38th day. By the 43rd day the shells of the ears were filled with high, fungating masses of growth, blocking the auditory

canals, and covered with thick, dirty brown scab, save along a macerating, longitudinal fold (Fig. 16). The ears were distorted, and nodular on their outer sides

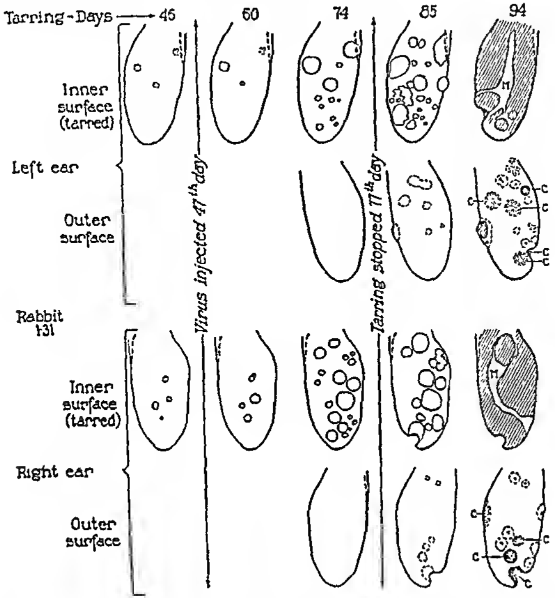

CHART 5. The course of events after virus injection into D.R. 1-31 (Experiment 2). Hatching shows areas of confluent proliferation. The broken lines indicate the ill defined edges of the mounds appearing on the outsides of the ears, cross-hatching there means ulceration, and stippling, subepidermal malignant growths. No attempt has been made to designate the malignant growths on the inner surface, because these were largely hidden in the confluent, fungoid masses. C, C = cancers on the outer surface, as disclosed by sections. M = furrow due to maceration.

(Fig. 17), and here the thickening (Y) was greater, and an ill defined bulge on the outside of the right ear near its base (Cs) first noted on the 28th day, had become prominent, 2½ cm. across, and fluctuating.

The animal was chloroformed on the 48th day, when moribund. On cutting through the ears a thick layer of soft tissue was disclosed, covering the insides, mostly of pinkish cream color but with gray streaks and patches (Fig. 18 *a*). There was some vertical striation but much diversity of texture, owing to imbedded growths, once discrete and still discernible by reason of their irregular markings and serpiginous or dotted necrosis. Many had extended through the cartilaginous plate,—especially near the ear tips where lacunae were frequent,—giving rise to the mounds and ulcers on the outer side. Also present here were scattered, discrete, acorn-shaped growths, creamy or dark gray, with a fine, vertical striation (Fig. 18 *b*),—later stages of the breast-shaped, subepidermal mounds previously noted. They were papillomas such as are caused by the Shope virus, and had originated deep in the skin and dried into blunt, dark cones at their summits. The deep thickening (Y) consisted of papilloma tissue of similar sort that had never erupted.

Blocks were fixed from 20 different situations, mostly where growths had extended through the cartilage. At 12 of them one or more destructive, infiltrative growths were found, squamous cell carcinomas histologically (Figs. 21, 23). All were of considerable size, 1.5 to 4 cm. in diameter, search disclosing none that was just beginning. The bulge on the outside of the left ear (Cs, Figs. 16 and 17) proved to be a lenticular cyst (Fig. 22) full of thin fluid containing necrotic fragments. Its ragged walls were lined with anaplastic tissue of carcinomatous character (Fig. 23), and similar tissue, largely destroyed by purulent infection, was present on the inner surface of the ear opposite it (Fig. 22).

The thick layer covering the inside of the ears consisted for the most part of papillomatous tissue, predominantly of ordinary virus type, though with discrete "papillomas of the second order" (2), cystic papillomas, malignant papillomas, and growths with the morphology of frank carcinomas incorporated in it, as were also a few old, fibrous, more or less pedunculated tar papillomas.

In one of the swollen, auricular lymph glands 2 minute epithelial growths were found, one keratinizing, and of squamous cell type, the other wholly anaplastic. There were many small abscesses in these glands and in the lungs.

D. R. 1-35 N, previously tarred 46 days, had at injection one wart 4 mm. across which did not alter later, though another small growth appeared by the 28th day. Not until nearly 40 days had elapsed did more develop. Then they arose rapidly as numerous, scattered cauliflowers, cones, or onions on the inner surface, and mounds or acorns on the outer side. Nearly all were slaty gray. Tarring was kept up until the 64th day. Under its influence many of the growths became large, and a few on the inner surface discoid and ruddy. On the 81st day the rabbit was killed because moribund from sepsis. The growths had continued to prosper, yet none had extended through the cartilage and only papillomas were found in the numerous blocks taken.

In one of the rabbits of this experiment the injection resulted in a profuse localization of virus in the ears, as attested by the develop-

ment of innumerable papillomas together with many growths that appeared to be cancers and behaved as such during the few weeks before death. Were it not for the results of Experiment 1 and for the findings in the other animal receiving the inoculum, one might be tempted to suppose that the preliminary incubation of the virus with heated cancer extract had resulted in a carcinogenic material of singular potency. In this animal, however, only papillomas occurred, although tarring was kept up for more than 2 months after the virus injection, with the aim of inducing secondary cancerous changes in some of the many, vigorously proliferating growths.

The Changes Caused by the Virus in Skin Long Tarred

It seemed well to extend the observations with the same virus material (W.R. 1211) to rabbits tarred for much longer periods. 2 were available that had been tarred over the entire inner surface of the ears for 40 weeks, and carried in consequence numerous, very large, cauliflower growths and fleshy, rounded tumors with more or less pedunculation, all appearing benign.

Experiment 3.—The rabbits were injected with 15 cc. of 2 per cent filtered virus fluid and two weeks later tarring was stopped. *D.R. 6-46* had at injection 15 warts up to 3 cm. across and more than 2 cm. high. None was gray. 17 days later a few gray, subepidermal mounds had appeared on the outer sides of the ears, and the biggest growth on the inside, previously a smooth, ruddy, almost tangential sphere, showed several protruding bosses on its surface, which in another 3 days had become large, rugose, and patched with gray (Fig. 30). Many small, gray papillomas had appeared elsewhere on the inner side of the ears, and on the outer side at one edge a scabbed thickening had developed, more than 1 cm. away from any other lesion. After another 4 days this had become a raised, ulcerated disc, 1.3 cm. across, with a smaller, slightly raised scab opposite it on the inside. It looked cancerous.

During the next 7 weeks the new growths on the insides of the ears enlarged rapidly, became crowded and in some regions confluent. Where discrete they were cone- or onion-shaped, gray or infrequently pink. They encroached upon and obscured all of the tar warts except the large pedunculated sphere studded with bosses. This was snipped off on the 36th day, and was found to consist mainly of fibrous tissue, covered almost everywhere with thick, papillomatous epithelium, some of it melanotic. There were some gray or creamy "acorns" embedded in the growth, and in a few places its covering was mere hyperplastic

epidermis. Many gray papillomas had appeared in the hyperkeratotic skin on the back of the neck.

The rabbit was killed on the 73rd day after injection, while still in excellent condition. The growth that looked cancerous had ulcerated further, causing necrosis of the cartilage and a deep nick in the ear. It had extended to a distance of about 1.3 cm. from this nick on either side of the cartilage, and was erosive, not fleshy. Microscopically, it was a squamous cell carcinoma, anaplastic in some regions. No malignant changes were evident elsewhere. Both ears were almost completely covered on the inner surface with pink or gray growths, and many of the latter were present on the outer surface as well. The regional lymph glands were not examined.

D.R. 6-51 had six large, pink, tar tumors at time of injection, several of them 3 to 4 cm. in diameter, fleshy and pedunculated. When tarring was stopped 14 days later, some scattered, small, gray mounds had just appeared on the outer surface of the ears; and in another 3 days these organs were noted to have undergone the brawny, hot thickening and stiffening already described. A raised, scabbed disc had formed in the hyperkeratotic skin of the outside of one ear, more than 2 cm. away from the nearest growth, and a mound had appeared opposite a tar tumor on the inside. The surface of several of the larger tar tumors had become nodular, and elsewhere on the inner surface many new, pink, discoid tumors had arisen. All enlarged rapidly. By the 21st day the ears were greatly thickened, distorted, hot, heavy, and pendulous. The changes continued; the ears became several centimeters thick; nodular bulgings appeared on their outer surface, subepidermal growths along their edges (Fig. 25); their orifices became choked with pultaceous matter, and their concavities full of confluent growth, partly obscuring the tar tumors (Fig. 24). On the hyperkeratotic back of the neck numerous gray papillomas appeared.

The animal died of seropurulent pleural effusions on the 32nd day. The ears weighed 220 gm. (Fig. 25). On the inner side of the cartilage was an irregular sheet of vertically striated tissue 8 to 12 mm. thick; and a similar sheet, mostly subepidermal, existed outside. Most of the old tar tumors had been destroyed by maceration. The scabbed disc had grown smaller: it proved due to a papilloma of virus type. No malignant growth was found elsewhere on extensive sectioning.

The virus localized abundantly in the ears of these rabbits. In view of the marked general changes, the numerous, large, tar warts, and the outcome of the previous experiments, it had seemed reasonable to suppose that many malignant growths would be elicited. On the contrary, only one appeared after the injection. Yet the conditions were so favorable to the virus, that the ears of one animal reached enormous size within a few weeks, owing to confluent papillomatous proliferation.

Later Course of the Induced Malignant Tumors

Most of the rabbits in which the virus caused confluent or coalescing growths died early from sepsis incidental to their presence. Metastases had already developed in the lymph nodes of 2, but they might have arisen from cells thrust into the lymphatics when the ears were biopsied. In the next experiment the inoculum was reduced to elicit fewer growths; and these were allowed to run their course without interference. The virus material W.R. 1211 was by now exhausted, and recourse was had to that from W.R. 1183.

Experiment 4.—The 42 rabbits had been tarred for 89 days over about half of the inner surface of the ears. At injection they were separated into four comparable groups. The ears of some were but little changed, and carried no warts or only one or two; but those of more than half of each group were thickened, hyperkeratotic and hot, and bore several warts.

Eight rabbits were injected intravenously with 15 cc. of 0.5 per cent filtered virus fluid, and some of it was tattooed into an area 2 to 3 cm. across on the shaved, left side of the body. Tarring was kept up for 25 days more. All of the tattoo inoculations yielded papillomas, and in 5 of the 8 animals virus localized in the tarred skin as proven by numerous sooty papillomas on both aspects of the ears. In 3 of the 5 many rapidly enlarging, ruddy tumors also appeared on the inner surface, and in 2 of the 3 some of the growths here were malignant. A second group of 9 rabbits were similarly injected but tarred no longer. Though they were susceptible, as shown by growths at the tattoo sites, relatively few sooty papillomas developed on their ears, only occasional ruddy growths, and no malignant tumors. A third group, of 11 animals, received 0.2 per cent virus and were tarred later, like the first lot. Though they all developed tattoo growths very little virus came out into the ears, few gray papillomas arising, only an occasional ruddy growth, and none that was malignant. This group can be thought of as furnishing accessory controls. The 10 controls proper were tarred like the first and third lot and most of them were kept for months after the termination of the experiment. Their warts remained small and either disappeared later on, persisted as such, or very slowly enlarged.

In a succeeding paper, the findings will be scrutinized in detail. Here only those animals of the first group that developed malignant growths need be considered. A staphylococcus meningitis caused death of one (No. 28) on the 71st day. It had no warts when injected, nor any 2 weeks later, but during the 3rd week, when virus papillomas were appearing on the side, many discrete growths arose on the ears, and after another 2 weeks more than 50 were present, mostly sooty and still small. At the charting of the 36th day a new, pink, subepidermal mound attracted attention. It was 6 mm. across, had encroached upon a neighboring, sooty papilloma, appeared to be infiltrating laterally, had a scabbed top,

and was recorded as probably malignant. A week later a firm mound had developed opposite it on the outside of the ear. It grew steadily, infiltrating and ulcerating on both aspects of the organ, and when the animal died was 2 cm. in diameter (Fig. 28). On the outer side several fleshy, subepidermal prongs, not visible in the photograph, extended from it toward the base of the ear. Such prong-like extensions have never been found extending from the tar carcinoids of our many control rabbits, nor have these grown after tarring was stopped. The microscope showed a growth with the histology of a squamous cell carcinoma with elongated, cystic extensions such as are encountered in many cancers derived from ordinary, virus-induced papillomas (5). It will be pictured in a later paper.

The other rabbit developing malignant tumors (No. 27), had 3 small warts at injection. During the 3rd week thereafter growths suddenly appeared in considerable number on the ears, nearly all on the left, some pink, the majority gray; and papillomas were now visible at the tattoo site. During the next 2 weeks many growths developed on the left ear and a few on the right. On the 36th day a firm thickening could be felt on the outer side of the right ear opposite a fleshy disc with ill defined margins that dated from the 3rd week after injection. The disc soon became a broad, weeping mound which encroached upon and undermined the nearest growth, a sooty papilloma originally more than 1 cm. away (Fig. 31); and the extension to the outer side of the ear, elsewhere wholly devoid of tumors, underwent ulceration. The ulcer had raised, infiltrating edges (Fig. 32). At the 123rd day the malignant growth had long since destroyed the neighboring, sooty papilloma on the inside, as well as others further off and a considerable part of the ear itself (Fig. 33). The foul, granulating expanse was stippled with yellow dots, suggestive of keratinization. The ear had reverted to the normal in the region not occupied by growths.

The enlarging tumors on the left ear soon became crowded, and maceration took place along its middle. Here, where the tar had been directly applied, no gray growths arose but instead several weeping, pink discs that rapidly grew large and fleshy. Owing to later distortion these came to occupy the bottom of a deep, longitudinal fold, with many high, crowded, gray papillomas fencing them from close inspection. On the 70th day a mound had appeared outside the cartilage, opposite one of them, and a week later 2 more mounds opposite others. By now many large, conical or jagged papillomas, more or less confluent and mostly sooty, were present on both sides of the ear.

On the 93rd day the lymph glands at the base of both ears were enlarged and firm, and 2 weeks later a nodule nearly 1 cm. across was palpable in a gland on the left, now 3 cm. long, and another on the right, 0.5 cm. in diameter. The nodules enlarged rapidly and others appeared in neighboring glands. Figs. 14, 33, and 34 tell the state of affairs on the 123rd day. The ears had undergone extensive destruction. By the 156th day about half of the right one was gone (Figs. 35, 36), and only the stump of the left remained, thickened, brawny, and edematous. Enormous metastatic masses had replaced the auricular nodes, and others

existed lower down in the neck. A thick cord could be felt connecting the growth on the right ear with the nearest glandular mass. The skin over the most prominent of those on the left side was attached, and fluctuation could be felt immediately beneath it.

The thin, weak animal was killed on the 159th day. Microscopically the destructive growths on the ears had the morphology of more or less anaplastic, squamous cell carcinomas (*Fig. 37*), and the masses in the neck consisted of similar tissue, with remnants of the lymph nodes and of the adjacent salivary glands (Figs. 15, 29), amidst abundant, reactive connective tissue. The fluctuation was due to an abscess amidst the neoplastic tissue. The cord connecting with the growth on the right ear was carcinomatous. No visceral metastases were found, but much amyloid change in liver and spleen.

In this experiment reducing the amount of virus injected had one of the desired results, premature death from sepsis being much less frequent; but the reduction was carried too far in one group of animals, with the result that very few virus localizations occurred in their ears. When many took place the papillomas still appeared relatively late and grew rather slowly as compared with those of the previous experiments. 5 of the 8 rabbits receiving the largest inoculum and tarred for 25 later days developed sooty growths on the ears in moderate number, with some pink ones; and concurrently malignant tumors appeared in 2 of the 5 individuals. One died early of intercurrent causes. A notably invasive growth arose on the right ear of the other, as also a few sooty papillomas, while on the left ear several malignant growths of the same sort appeared, together with numerous papillomas. Both ears underwent progressive destruction and immense metastatic masses formed in the regional glands. The associated papillomas remained merely such while this was happening.

The Effect on Tar Warts of Virus-Induced Fibromatosis

Castiglioni (13) has reported that rabbits rendered syphilitic by the intravenous route are notably responsive to tarring, although the ears show no signs of syphilitic infection. He describes both papillomas and carcinoids as appearing early, but no cancers arose. It has seemed possible that some of the growths elicited in the present work might be tar warts stimulated to factitious malignancy (14) by connective tissue disturbances referable to the virus, although no such disturbances have been found, and the hypothesis will not explain the

metastasizing tumors or those which continued malignant while round about them the ears were reverting to normal. Nevertheless tests were undertaken with the virus causing rabbit "fibromas" (15) to find what effect a vigorous connective tissue proliferation would have upon tar warts.

A 5 per cent extract of glycerolated "fibroma" tissue[2] in Tyrode was cleared with the centrifuge, and 0.1 to 0.3 cc. was injected at each of 4 to 16 situations in the skin of the inner surface of the ears of 4 rabbits. The ears of 3 had been tarred twice a week for 165 days, and carried 4 to 7 warts from 3 to 25 mm. in diameter, while those of the fourth had been tarred thrice weekly for 5 months, and, after 6 months' intermission, for 5 months more twice weekly, with result in 29 warts 3 to 10 mm. across. The virus fluid was injected directly under or next to several warts of each animal, and the tarring was kept up afterwards for 4 weeks. Early in the 3rd week "fibromas" developed as ruddy mounds or discs, but no new warts were evoked. Repeated punch biopsies were made. Often the fibromatous proliferation took place immediately under the epithelium of the tar tumors, yet the latter did not extend downwards, the only effect upon them being pressure distortion or occasional local necrosis. Frequently the "fibromas" extended through the cartilaginous plate, with result in low mounds beneath the outer skin. Here too no epithelial downgrowth was induced.

The Findings as a Whole

The present paper is mainly concerned with the gross changes taking place in the tarred skin after the virus lodged in it, and with those tumors which appeared to be malignant. A variety of other growths were also elicited in addition to the papillomas characteristic of the virus, namely papillomas of complicated pattern and problematic malignancy, cystic papillomas, and frankly malignant papillomas. They will be considered in a later paper. The unavoidable employment of several virus materials added a complicating factor to the many implicit in the experiments; yet the results are consistent.

In the individuals most susceptible to the virus, its localization at numerous situations in the tarred ears was signalized by a sudden, brawny, warm thickening which occurred in the 3rd week after the injection, an incubation period roughly corresponding with that when a virus fluid of moderate pathogenicity is rubbed into scarified normal skin. During the next few days a more or less confluent

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[2] Strain D, D.R. 1514, kindly provided by Dr. Shope.

sheet of papilloma tissue formed on the inside of the ears, and low, scattered, subepidermal mounds, mostly gray but occasionally pink or creamy, appeared in the hyperkeratotic skin of the outer side. At the same time some of the pre-existing tar warts, previously indolent, began to enlarge with great rapidity, and fleshy, pink or cream-colored growths also arose where no warts had been present. That these were nearly all referable to the action of the virus was plain from the findings in the controls; yet all resembled in their gross appearance one or another of the various tumors that follow merely upon tarring, though they proliferated much more vigorously. Some were mounds or discs, fleshy cones or dry, cutaneous horns; others were of cauliflower, onion, or hassock shape; while yet others became fleshy, fibrous spheres that underwent pedunculation secondarily.

In many cases the mounds forming on the outside of the ear rapidly became breast-shaped, with nipple-like, dark, keratinized protrusions which later heightened into vertically striated cones, the growths then taking on the aspect of ordinary, virus-induced papillomas. In some animals, though, they remained predominantly subepidermal, and, coalescing, formed plateaus covered with rounded protrusions (Fig. 3). Many growths on the inside of the ears were lost to view in the sheet of papillomatous tissue that rose about them, but others maintained their identity until the aural shell filled up with foul, macerating tissue and its whole interior became a fleshy, scabbed mass (Figs. 16, 17). Then, on cutting through the ear, the sheet of new tissue showed some vertical striation, perhaps streaked or mottled in gray, the pink growths amidst it presenting a diversity of patterns (Fig. 18 *a*).

Special significance attaches to these rapidly enlarging tumors which took the form of low mounds or raw, beefy discs dotted with yellow necroses; for many of these proved malignant. They had ill defined margins, or a rim of raised and infiltrated skin, and sometimes a depressed, crateriform center. Some were derived from tar warts, but others arose where none had been visible. As a rule they enlarged progressively, even though tarring was stopped; invaded and replaced the tissue about them; and soon extended through the cartilage, causing ulceration on the outer side.

Death occurred early, owing to the septic state of the ears, which were full of foul, fungating growth, yet metastasis had already occurred in some cases. In one of these the secondary nodule was recognized only 32 days after the parent growth on the ear had first declared itself, 18 days after virus injection. Both grew rapidly.

When less virus localized in the ears, as attested by a smaller number of sooty papillomas, the growths of other sorts were less numerous. They arose concurrently with the crop of papillomas, and at about the same time some of the tar warts started to grow at an unprecedented rate. The appearance of one or more gray, subepidermal mounds on the outer surface of the ears often served as a tell-tale to the presence of the virus when it was proving effective at but few situations. In such instances some gray or almost black growths usually arose on the inner

surface as well, but here the melanoblasts responsible for their hue (16) were less frequent and the epidermal alterations were greater. The more pronounced these alterations the more frequent in general were non-pigmented growths.

Generally speaking, malignant growths were few as compared with ordinary virus-induced papillomas; and in proportion as the amount of virus acting upon the ears was diminished, they became rare. One noteworthy exception to the rule was met, however, several cancers arising in rabbit 15 of Experiment 1 at the same time as did thinly scattered papillomas (Figs. 6 to 13). Only animals favorable to the virus, as evidenced by the induced papillomatosis, developed malignant tumors also; yet they failed to occur in some individuals so favorable that their ears were largely converted into papillomatous masses (Figs. 24 and 25). The malignancy was often multiple, and involved both ears.

The stimulating effect of tar upon the growths that it elicits is one of the truisms of cancer research. Virus-induced papillomas are also very responsive to its influence, often burrowing, extending through the cartilaginous plate, and ulcerating, though remaining essentially benign, as shown by the fact that they build up later into discrete conical or onion-shaped growths of the characteristic sort. Needless to say the tarring after virus injection must have elicited some tumors referable merely to it, though few such developed in the controls. The contrast afforded by these latter animals was remarkable.

A secondary resistance to the sooty papillomas occasionally developed, as evidenced by the retrogression of most of them; and under its influence some of the pink growths that had been growing rapidly also disappeared, or reverted to their previous indolent state. This happened in rabbit 15 of Experiment 1. Yet while most of the papillomatous growths elicited by the virus in this animal were disappearing the malignant tumors continued to proliferate and invade, and a few papillomatous growths also went on enlarging rapidly (Chart 2).

Some tar usually reached the base of the neck where the ears rested, producing alopecia and hyperkeratosis; and many growths sometimes

appeared there after the virus injection. They were always gray or pink papillomas of the characteristic sort.

DISCUSSION

Were the malignant growths which arose in the tarred skin referable to the action of the virus? In only one of more than 90 tarred but uninoculated rabbits, of the breed experimented upon, many of them tarred for long periods, has any such growth arisen. It was a metastasizing squamous cell cancer consequent upon tarring for two periods of 6 months and 5 months respectively, and it appeared after 21 months in all. When the virus failed to localize in the ears of the injected rabbits no malignant growths developed; and the more abundant its localization as evidenced by gray papillomas engendered, the more often, generally speaking, did they occur. They appeared at the same time as these papillomas, that is to say a few weeks after injection of the virus; and they arose only when conditions were favorable to the latter, as demonstrated by the behavior of the gray growths. They occurred with a frequency unparalleled in the recorded experience with tarred rabbits, and were often multiple, and frequently numerous, facts which will find further exemplification in a succeeding paper. In sum, the facts leave no doubt that the virus called them forth.

Were the malignant growths the expression of an unique disease, simulating cancer but to be discriminated from it? A categorical answer can be given to this question, owing to the fact that tumors as a class inscribe themselves upon the organism with a minute elaboration. The malignant growths of the present work exhibited all those histological features which typify carcinomas of squamous cell origin; yet such features do not suffice in themselves for a diagnosis of cancer in the case of growths arising in tarred skin. These may be highly anaplastic and invade rapidly, penetrate through lacunae in the cartilage and cause ulceration, yet disappear after a time or undergo a transformation into indolent papillomas, even when tarring is kept up (9). Some of the tar tumors of our control animals were of this sort, incapable of independent malignancy, as shown by their disappearance or reversion to the benign state when tarring was left

off. Final proof that the malignant growths evoked by the virus were true carcinomas rests upon those cases in which the growths not only had the morphology but manifested the independent activity of such tumors. This happened in every instance save one, when the animal lived long enough, and in this one the growth was destroyed by purulence after biopsy (Experiment 3).

Since the publication of a preliminary report of the work here set forth, Lacassagne and Nyka (17), using benzpyrene instead of tar, have confirmed its findings, and Andrewes, Ahlström, Foulds, and Gye (18) have studied the alterative effects of tar upon the outcome of infection with the virus which gives rise to rabbit "fibromas." Ordinarily this virus causes connective tissue growths which are restricted to the immediate site of inoculation and regularly retrogress after a few weeks of active proliferation. This was the case with the strain employed by the authors mentioned. But when the animal had received an intramuscular injection of tar, and the virus was thrown into the blood stream, not only did it elicit in some cases widely distributed growths with the character of "fibromas," which enlarged progressively and caused death, but in certain instances growths arose which the authors describe as of neoplastic character. They appeared at the site of the tar injection, that is to say, where the connective tissue had undergone most change. In this general relation it is important to recall the demonstration of Teague and Goodpasture (19) that the virus of herpes simplex, when acting upon tarred skin, induces lesions resembling those of herpes zoster.

SUMMARY

The Shope papilloma virus elicits carcinomas forthwith, as well as papillomas in great variety, when it is distributed by way of the blood stream to the tarred epidermis of domestic rabbits.

The phenomenon will be analyzed in succeeding papers with the aid of additional instances.

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EXPLANATION OF PLATES

All of the sections were stained with methylene blue and eosin.

PLATE 13

FIG. 1. Early malignant downgrowth in rabbit 11 of Experiment 1: 22nd day after inoculation. The surface epithelium from which the anaplastic proliferation derives, though still intact, stains much lighter than that adjoining it. ×44.

FIG. 2. Ulcerating, destructive growth at the ear margin of rabbit 12: biopsy specimen of the 29th day. The anaplastic proliferation has extended beneath the skin, past the cartilage, and under an adjacent, newly appeared papilloma at the ear edge. ×12½.

FIG. 3. Ears of rabbit 12 on the 43rd day after virus inoculation: to show some of the malignant growths (C, C), and the mamelonated plateaus of papillomatous growth, largely subepidermal. The malignant ulceration has eaten deep into the tip of the left ear. (The aural shells were full of coalesced, fungating tissue,—*vide* Fig. 16 of another animal.) ×2/5.

FIG. 4. Autopsy specimen from rabbit 12: Part of a large, ulcerated, malignant growth with papillomatous features. Extension has taken place through many lacunae in the cartilage. At the right there is heavily pigmented, benign papillomatosis. The keratin overlying it has been cut away. ×11.

Photographed by Joseph B. Haulenbeck and Louis Schmidt

(Rous and Kidd: Carcinogenic effect of papilloma virus. I)

Plate 14

Fig. 5. Edge of a cystic, metastatic growth in an auricular node of rabbit 12. (See also Fig. 19.) × 78.

Figs. 6 to 13. The ears of rabbit 15. Figs. 6 and 7, 8 and 9 show the inside and outside of the right and left ears respectively, on the 41st day. Figs. 10 and 11, 12 and 13 show the state of affairs 16 days later. The hole in the left ear (Fig. 8) was due to a biopsy on the 29th day. It later filled with growth (Fig. 12). C, C= carcinomas, as determined microscopically. P, P = the aggressive papillomas described in the text. The scattered small growths were all ordinary papillomas, mostly dark gray.

Fig. 14. Ears of rabbit 28 (Experiment 4) on the 123rd day (see also Figs. 33 and 34). The ulcerated, coalesced, malignant growths on the left ear, which have extended through and destroyed its central portion, are surrounded by numerous, discrete, sooty papillomas. A similar malignant tumor has perforated the right ear and extended to the outer side which is devoid of growths elsewhere. There is metastatic enlargement of the auricular lymph nodes (arrows). × 1/3.

Fig. 15. Metastasis in an auricular lymph node of the same rabbit. × 41.

Photographed by Joseph B. Haulenbeek and Louis Schmidt

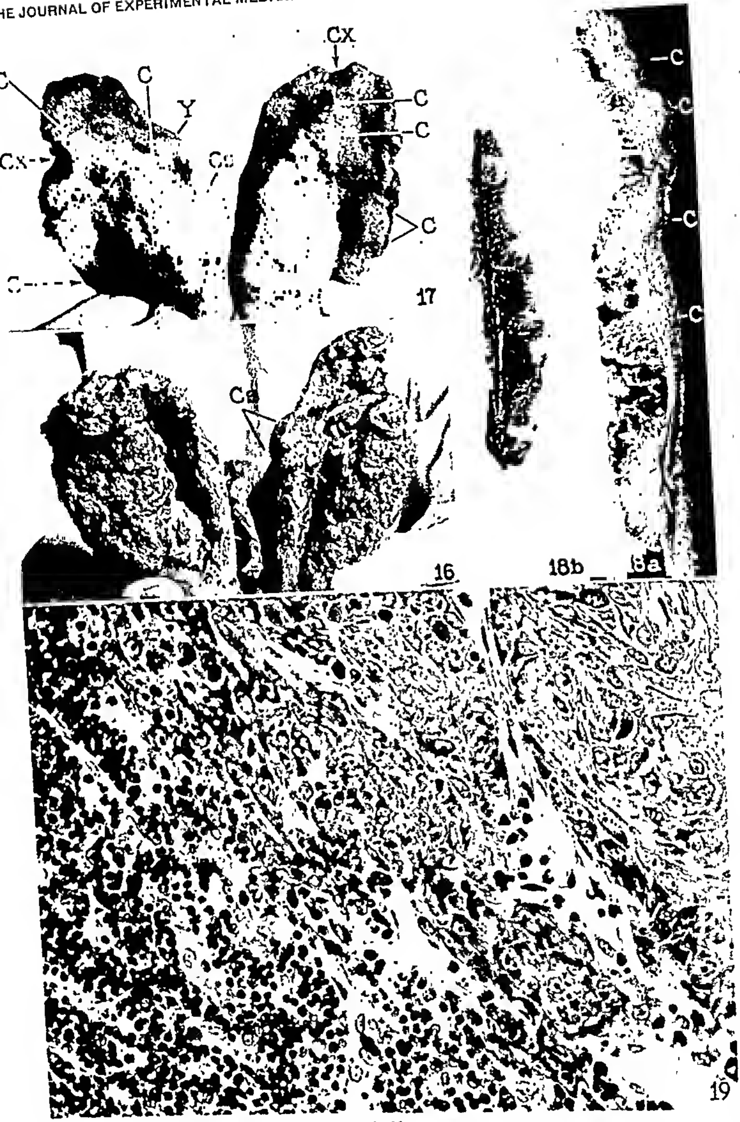
(Rous and Kidd: Carcinogenic effect of papilloma virus. I

Plate 15

Figs. 16 and 17. The ears of D. R. 1-31 (Experiment 2) on the 47th day. The aural concavities are full of confluent, scabbed, fungating growth, and great distortion exists. On the outer side are scattered mounds and bulgings, some being deep lying, benign papillomas capped with dark keratin, while others are malignant (C,C) and in some cases ulcerated. Two biopsies (Cx) had been made at the ear margin. At Y is the diffuse thickening mentioned in the text (page 415), and at Cs the bulge caused by a deep lying, cystic, malignant growth (*vide* Fig. 22). $\times$ 2/5.

Fig. 18. (*a*) Longitudinal slice through an ear of Figs. 16 and 17. The diversified markings of the fungoid tissue indicate the variety of the growths. At several places (C,C) they have extended through the cartilage and formed mounds on its outside, especially near the ear tip. (*b*) Slice through several of the embedded, acorn-shaped papillomas on the outer side of the ear. 2 are medium and dark gray respectively, and all are vertically striated. $\times$ 4/5.

Fig. 19. Invasion by the glandular metastasis shown in Fig. 5. $\times$ 400.



Photographed by Joseph B. Haulenbeek and Louis Schmidt

(Rous and Kidd: Carcinogenic effect of papilloma virus. I)

PLATE 16

FIG. 20. Extension of an anaplastic growth through a lacuna in the ear cartilage: biopsy on the 38th day, rabbit 1-31 (Experiment 2).

FIG. 21. Random section through the edge of an ear, at autopsy of the same animal. A highly anaplastic, ulcerating growth has extended through the cartilaginous plate. Nearby is a deep lying, heavily pigmented, acorn-shaped papilloma. × 10.

FIG. 22. Cross section of the bulging cyst, Cs, of Fig. 17. It is lined with malignant growth (Fig. 23), which can be seen also in a broad, ulcerated expanse opposite it on the inner side of the ear. × 4.

FIG. 23. Part of the cyst wall. × 40.

Photographed by Louis Schmidt

(Rous and Kidd: Carcinogenic effect of papilloma virus. I)

Plate 17

Fig. 24. Results of a profuse virus infection of skin tarred for many months previously (rabbit 6-51, Experiment 3: 23rd day after virus injection). Within the greatly thickened aural shells 2 large tar tumors can still be seen amidst much new-formed, fungating tissue. $\times$ 2/5.

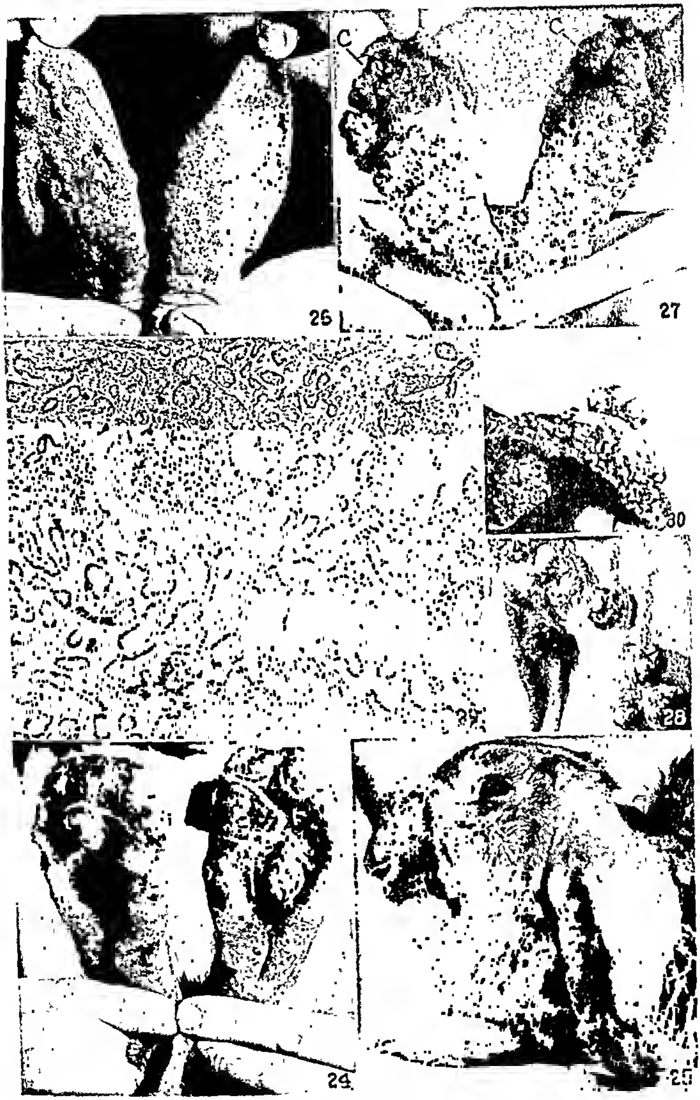
Fig. 25. Same ears on the 28th day. They are distorted and rendered enormous by diffuse, papillomatous proliferation, which is almost entirely subepidermal on their outer side and along their edges. They weighed 220 gm. Nowhere did malignancy exist. $\times$ 2/5.

Figs. 26 and 27. Ears of rabbit 12 on the 29th and 42nd days: to show the rapid progress of the changes. $\times$ 2/5.

Fig. 28. Ulcerated, malignant growth on the outside of the ear of rabbit 27 (Experiment 4): 67th day. The bracket indicates its situation. The other growths are gray papillomas. Shadows complicate the picture. $\times$ 4/5.

Fig. 29. Extension to a salivary gland of the metastatic tumor at the base of the right ear of rabbit 28 (Experiment 4). $\times$ 42.

Fig. 30. Virus-induced, bulging, papillomatous excrescences on an old, pedunculated tar wart. Some of them are patched with gray. The surface of the wart had previously been smooth (D. R. 6-46, Experiment 3: 36th day). $\times$ 2/5.



Photographed by Joseph B. Haulenbeek and Louis Schmidt

(Rous and Kidd: Carcinogenic effect of papilloma virus. I)

PLATE 18

FIG. 31. Malignant growth on the inner surface of the right ear of rabbit 28: 67th day. It has encroached upon a nearby papilloma of dark hue. × 2/5.

FIG. 32. Extension of the growth of Fig. 31 to the outer surface of the ear: 107th day. × 2/5.

FIG. 33. Same growth on inner surface, with metastases in the auricular glands: 123rd day. (See also Fig. 14.) × 2/5.

FIG. 34. Destructive growths on the other ear of the rabbit, with glandular metastases: 123rd day. (See Fig. 14.) × 2/5.

FIG. 35. Left ear of Fig. 34 on the 156th day. Only an edematous, ulcerated stump remains, covered with dry blood. There is swelling round about and a large metastatic mass at the angle of the jaw. × 1/5.

FIG. 36. The ears and metastatic masses from behind: 156th day. × 1/5.

FIG. 37. Section through stump of the left ear: 159th day. × 11.

Photographed by Joseph B. Haulenbeck and Louis Schmidt

(Rous and Kidd: Carcinogenic effect of papilloma virus. I)

A GROWTH INHIBITORY SUBSTANCE FOR THE INFLUENZA GROUP OF ORGANISMS IN THE BLOOD OF VARIOUS ANIMAL SPECIES

THE USE OF THE BLOOD OF VARIOUS ANIMALS AS A SELECTIVE MEDIUM FOR THE DETECTION OF HEMOLYTIC STREPTOCOCCI IN THROAT CULTURES*

BY ELMA KRUMWIEDE AND ANN G. KUTTNER, M.D.

(*From Irvington House, Irvington-on-Hudson, New York*)

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The isolation of hemolytic streptococci from throat cultures is often rendered tedious and difficult by the presence of the so called bacillus X or *Hemophilus hemolyticus*, first described in 1919 by Pritchett and Stillman (1). Mueller and Whitman (2), in making routine throat cultures on horse blood agar plates, found it impossible to differentiate the colonies of these hemolytic bacilli on a crowded plate from those of beta hemolytic streptococci. These authors devised a special method for eliminating bacillus X by placing the swabs in alkalinized broth from 1 to 2 hours before plating.

The bacillus X described by Pritchett and Stillman (1) and further studied by Stillman and Bourn (3) is strictly hemophilic, requiring for growth both the heat-stable "X" factor associated with hematin and the vitamin-like "V" factor found in blood and plant cells. On blood agar this organism is readily differentiated from *Hemophilus influenzae* by its hemolytic property, but on oleate or chocolate agar the colonies are indistinguishable.

Further studies of these hemolytic bacilli resembling *H. influenzae* were made by Rivers and his coworkers (4–6) and Fildes (7). Rivers found that these organisms differ in their growth requirements, some requiring both X and V, some only V, and some only X. He suggested the name *Bacillus parainfluenzae* for those bacilli requiring only the addition of V to the medium. Rivers described these organisms as

* This work was aided by a grant from the Commonwealth Fund.

forming slightly opaque hemolytic colonies, often firm enough to be pushed about on the surface of the agar. He did not mention variations in the colony size. He stated that in fluid media flocculi similar to those seen in streptococcus cultures were usually formed. Upon microscopic examination the bacilli were very pleomorphic and tended to be larger than *H. influenzae*. Rivers noted that stock cultures of these organisms tended to die out very quickly.

EXPERIMENTAL

In making routine throat cultures for the isolation of hemolytic streptococci, it has been found that many children in this institution carry large numbers of hemolytic bacilli in their throats. These bacilli are of two varieties: one forming a moderately large colony about 1 to 2 mm. in diameter surrounded by a fairly wide zone of hemolysis, the other a much smaller colony very similar to that of *H. influenzae* except for a small zone of hemolysis. Microscopic examination of the bacilli obtained from these two types of colonies showed that they are Gram-negative, non-motile, pleomorphic bacilli. The organisms obtained from the larger colony tend to be somewhat longer than those from the smaller colony. Both varieties produce hemolysis when grown in rabbit blood broth. The larger bacilli form flocculi in fluid media, whereas the small bacilli usually grow diffusely. Both types stain irregularly. Stock cultures die out quickly and are best maintained by daily transplants.

It has been found that the bacilli forming the larger colony in most instances require only V as an accessory growth factor and correspond to Rivers' *B. parainfluenzae*. The majority of the strains forming the small type of colony require both X and V and correspond to bacillus X (*H. hemolyticus*) of Pritchett and Stillman.

The presence of these two types of hemolytic bacilli in the flora of the throats of most of our patients has made the examination of rabbit blood agar plates unsatisfactory for the isolation of beta hemolytic streptococci. At the time that we began this study we were unaware of the work of Mueller and Whitman. An effort was made to devise a selective medium which would eliminate the hemolytic bacilli and allow beta hemolytic streptococci to grow. The addition of potassium tellurite to rabbit blood agar plates was tried. It was found that potassium tellurite, even in high dilution, hemolyzes rabbit cells, and the medium is therefore useless for the detection of hemolytic streptococcus colonies. Since potassium tellurite does not hemolyze sheep cells, sheep blood was substituted for rabbit blood.

It was found that when sheep blood is used, even without the addition of potassium tellurite, both varieties of hemolytic bacilli either fail to grow or grow very poorly with little or no hemolysis. Hemolytic streptococci on the other hand grow well on sheep blood.

Brown (8) compared the appearance of beta hemolytic streptococci on human, rabbit and horse blood, and found no essential differences as far as true hemolytic streptococci were concerned. He did not include sheep blood in his studies.

In order to be sure that typical hemolytic colonies are formed on sheep blood, the growth of several recently isolated strains of beta hemolytic streptococci has been compared on rabbit and sheep blood by surface streaking and pour plates. No difference in the number or size of the colonies has been observed, and the zones of hemolysis are comparable. It has been found that if the reaction of the agar base is slightly acid, pH 6.8, clearer zones of hemolysis are obtained than if agar with pH 7.4 is used.

Inhibitory Factor

The inhibition of the growth of the hemolytic bacilli on 5 per cent whole sheep blood plates has been so striking that it seemed of interest to try to analyze this phenomenon. The growth of two strains of the large hemolytic bacilli (*B. parainfluenzae*) and two strains of the small hemolytic bacilli (*H. hemolyticus*) have been compared with that of a stock culture of *H. influenzae*[1] throughout the following experiments. These five cultures constitute the test organisms referred to below.

Methods

Daily transplants of the test organisms were made on rabbit or guinea pig blood agar plates. Tubes of trypsin broth containing small quantities of rabbit blood were inoculated from the plate and grown overnight. Large amounts, 0.3 to 0.5 cc., were then inoculated from these tubes to plain trypsin broth the next morning. The experimental plates were streaked from the plain trypsin broth cultures which had grown from 4 to 6 hours, or until definite cloudiness could be observed. The growth of the test organisms on sheep and rabbit blood agar plates made with agar, pH 6.8 and 7.4, was compared. No difference in the growth was observed due to variations in pH. Therefore the same agar base, bacto Difco dehydrated blood agar base pH 6.8 (beef heart infusion agar) was used throughout. None of the test organisms grow on the plain agar base. The agar, tubed in 15 cc. amounts, was melted and cooled, and the substances to be tested were added before the plates were poured. When the quantity of blood used was less than 0.1 cc., a preliminary dilution of 1:10 was made. The results were recorded after 24 hours' incubation and again after 48 hours'. Defibrinated bloods were used in every instance.

1. Growth on the Blood of Various Species of Animals.—Rivers (4, 9) studied the growth of *H. influenzae* and closely allied hemolytic bacilli on various kinds of

[1] This culture was obtained from the Department of Bacteriology, New York Post-Graduate Hospital and Medical School.

blood. He found that these organisms grow well on rabbit, cat, dog, and pigeon blood, whereas on human and hen blood growth is unsatisfactory.

In view of the striking difference found by us between rabbit and sheep blood, it was thought of interest to compare the blood of animals closely related to the rabbit, such as the guinea pig and the rat, and that of animals closely related to the sheep, such as the goat and the cow. Horse and human blood were included for comparison. It was found that both varieties of hemolytic bacilli and *H. influenzae* grow well on various kinds of rodent blood. 5 per cent whole rabbit, guinea pig, or rat blood agar plates give equally good growth. When 5 per cent whole sheep, goat, or cow blood is used, the hemolytic bacilli and *H. influenzae* fail to grow or grow very poorly. With 5 per cent whole horse blood the test organisms grow fairly well, but the colonies are smaller than on rodent blood. It had been noted by Rivers (9) that human blood is often inhibitory for *H. influenzae*. With 5 per cent whole human blood somewhat variable results are obtained, but the growth in the majority of instances is poor and is inferior to that obtained on rodent blood.

TABLE I

Growth of Bacillus parainfluenzae hemolyticus, Hemophilus hemolyticus, and Hemophilus influenzae on Whole Unheated Blood of Different Animals

Medium: 5 per cent blood agar plates.

Organisms	Rodents			Artiodactylae			Perisso-dactyla	Pri-mate
	Rabbit	Guinea pig	Rat	Sheep	Goat	Cow	Horse	Man
B. parainfluenzae hemolyticus..............	+++*	+++	+++	±	±	±	++	±
H. hemolyticus (bacillus X)...............	+++	+++	+++	±	±	±	++	±
H. influenzae..........	+++	+++	+++	+	+	+	++	+

*++++ indicates excellent growth.
+++ " good "
++ " fair "
+ " poor "
± " doubtful "
These symbols have the same significance in all the tables.

These results are summarized in Table I. It is of interest that animals of closely related species such as the rodents behave alike in allowing good growth, whereas the three artiodactylae, sheep, goat, and cow, all show marked inhibition.

2. Is the Inhibitory Factor in the Cells or in the Serum?—Red blood cells of the rabbit, guinea pig, sheep, goat, cow, and man were washed three times and made up to volume with physiological salt solution. Agar plates containing 5 per cent washed cells of the various kinds of blood give the same results in every instance

TABLE II

Comparison of Whole Blood and Washed Erythrocytes of Various Animals

Media: 5 per cent whole blood agar plates.
5 per cent washed cell agar plates.

Organisms	Whole rabbit blood	Washed rabbit cells	Whole guinea pig blood	Washed guinea pig cells	Whole sheep blood	Washed sheep cells	Whole goat blood	Washed goat cells	Whole cow blood	Washed cow cells	Whole human blood	Washed human cells
B. parainfluenzae hemolyticus	+++	+++	+++	+++	±	±	±	±	±	±	±	±
H. hemolyticus (bacillus X)......	+++	+++	+++	+++	±	±	±	±	±	±	±	±
H. influenzae...........	+++	+++	+++	+++	+	+	+	+	+	+	+	+

TABLE III

Comparison of Washed Intact Erythrocytes and Laked Erythrocytes of Different Animals

Medium: 5 per cent cell suspension or the equivalent amount of laked cells in agar.

Organisms	Intact rabbit cells	Laked rabbit cells	Intact guinea pig cells	Laked guinea pig cells	Intact sheep cells	Laked sheep cells	Intact goat cells	Laked goat cells	Intact cow cells	Laked cow cells	Intact human cells	Laked human cells
B. parainfluenzae hemolyticus..............	+++	+++	+++	+++	±	±	±	±	±	±	±	±
H. hemolyticus (bacillus X).	+++	+++	+++	+++	±	±	±	±	±	±	±	±
H. influenzae...........	+++	+++	+++	+++	+	+	+	+	+	+	+	+

as plates containing whole blood, indicating that the inhibitory factor resides in the erythrocytes of these animals.

3. Is the Inhibitory Factor Present in Laked Cells?—Red blood cells of the rabbit, guinea pig, sheep, goat, cow, and man were laked by replacing the serum with distilled water. Agar plates containing 5 per cent of the solution of laked cells give the same results as plates containing 5 per cent washed intact cells of these ani-

mals, indicating that the inhibitory factor is not destroyed by the disruption of the erythrocyte.

4. Can the Inhibitory Factor in Sheep Blood Be Overcome by the Addition of Excess V?—V was prepared from fresh yeast according to the directions of Thjötta and Avery (11). 100 gm. of fresh yeast were emulsified in 400 cc. distilled water and adjusted to pH 4.6. The suspension was boiled for 10 minutes and allowed to settle. The clear supernatant fluid was tested for sterility, decanted, and stored in the ice box. In some experiments the unneutralized supernatant fluid was added to the medium, in others the yeast extract was neutralized just before use. The same results have been obtained with the unneutralized yeast extract as with the neutralized. It has been found that 0.5 cc. of the yeast extract added to 15 cc. of agar containing a minimal quantity of X is more than sufficient to assure good growth of *H. influenzae*. The addition of 3 cc., or six times the required amount of the neutralized yeast extract, to 15 cc. of agar containing 5 per cent sheep blood fails to overcome the inhibitory action of the sheep blood. In subsequent experiments, in which minimal amounts of the sheep inhibitor were used, a large excess of V also failed to give growth of the test organisms.

5. What is the Minimal Quantity of Sheep, Goat, Cow, and Human Blood Which Will Inhibit?—Decreasing amounts of sheep, goat, and cow blood were titrated in 15 cc. amounts of agar. Since with the diminishing quantities of blood, the amount of V supplied might be inadequate, a constant amount of V in the form of yeast extract (0.5 to 15 cc. of agar) was added. At first minimal quantities of X were also added but it was found that even the smallest quantities of blood used always contain sufficient X.

Table IV shows the inhibitory action of sheep blood. It will be seen that a concentration of 0.3 per cent sheep blood (0.05 cc. added to 15 cc. agar) is usually sufficient to inhibit growth in the presence of the standard amount of V. If the concentration of sheep blood is reduced further to 0.15 per cent (0.025 cc. added to 15 cc. of agar) growth is obtained indicating that this amount of sheep blood does not contain enough of the inhibitory factor to prevent growth, but that a sufficient quantity of X is still present. Inhibition of growth of the test organisms has been obtained with the same quantities of cow and goat blood (0.05 cc. added to 15 cc. of agar).

Human Blood.—Several specimens of human blood obtained from children with rheumatic heart disease, both in the active and in the quiescent stage, were compared with blood obtained from normal adults for the presence of the inhibitory factor. No differences have been noted between these various kinds of human blood. Human blood contains less inhibitor than sheep blood. It requires 3.3 per cent or ten times as much human blood (0.5 cc. added to 15 cc. of agar) as sheep blood to inhibit growth.

6. Is the Inhibitory Factor Thermolabile?—Fleming (10) first pointed out that the growth of *H. influenzae* is enhanced when blood agar is heated so as to produce a chocolate color. Chocolate agar is usually made by heating blood agar to a temperature of 90°C. for a few minutes. The improved growth obtained on

chocolate agar has generally been attributed to the fact that the red cells are broken up and their modified contents distributed throughout the medium. Rivers (9) noted that, whereas *H. influenzae* and allied organisms grow poorly on unheated human blood, excellent growth is obtained on heated human blood.

The effect of heating rodent blood and the bloods of other animals was compared. Although good growth is obtained on unheated rodent blood agar, the growth is enhanced when this medium is made chocolate. When sheep and goat blood are used, the difference between heated and unheated blood is striking. In contrast to the marked inhibitory effect of the unheated bloods, excellent growth is obtained on 5 per cent sheep or goat blood agar which has been heated to 90°C. for a few minutes. With cow and human blood a similar result is obtained, but care must be taken to heat the blood agar mixture at 90°C. for 15 minutes.

7. *At What Temperature Is the Inhibitory Factor in Sheep and Cow Blood Destroyed?*—As stated above it was found that if sheep or goat blood is heated to 90°C. for a few moments to make it chocolate, good growth is obtained. Using

TABLE IV

Effect of Decreasing Amounts of Sheep Blood in Agar in the Presence of a Constant Amount of V

Organisms	5 per cent sheep blood	1 per cent sheep blood	0.5 per cent sheep blood	0.3 per cent sheep blood	0.15 per cent sheep blood	5 per cent rabbit blood
B. parainfluenzae hemolyticus.............	±	±	±	±	++	+++
H. hemolyticus (bacillus X)...............	±	±	±	±	++	+++
H. influenzae............................	+	+	+	+	++	+++

minimal quantities (0.05 to 15 cc. of agar) of sheep or cow blood, it was found that 68°C. for 30 minutes is sufficient to destroy the inhibitory factors. Exposure to 56°C. for 1 hour fails to reduce the inhibitory action with these two types of blood. Temperatures between 56°C. and 68°C. were not tried. The inhibitory action of human blood is also destroyed at 68°C. for 30 minutes (Table VI).

8. *Is It Possible to Destroy the Inhibitory Factor by Other Means: Acidification and Alkalinization?*—*Acidification.*—Fleming (10) found that if blood is digested with normal sulfuric acid and neutralized and a small amount of the fluid thus procured is added to the medium, good growth of *H. influenzae* is obtained. This observer did not state what kind of blood he used.

In these experiments sheep blood was diluted 1:10 with salt solution. N/1 HCl was then added until the solution turned brown and no pinkish tinge remained (about 0.1 cc. of acid per 1 cc. of diluted blood). The mixture was shaken and allowed to stand for 30 minutes at room temperature. It was then neutralized with N/1 NaOH. 0.5 cc. of the acidified and neutralized blood was added to 15 cc. of agar containing the standard amount of yeast extract and a plate poured.

TABLE V

Comparison of Unheated and Heated Blood of Various Animals

Media: 5 per cent blood agar plates.
5 per cent chocolate agar plates heated at 90°C. for 15 minutes.

Organisms	Rabbit		Guinea pig		Rat		Sheep		Goat		Cow		Horse		Man	
	Un-heated	Chocolate	Un-heated	Chocolate	Un-heated	Chocolate	Un-heated	Chocolate	Un-heated	Chocolate	Un-heated	Chocolate	Un-heated	Chocolate	Un-heated	Chocolate
B. parainfluenzae hemolyticus	+++	++++	+++	++++	+++	++++	±	++++	±	++++	±	++++	++	++++	±	++++
H. hemolyticus (bacillus X)	+++	++++	+++	++++	+++	++++	±	++++	±	++++	±	++++	++	++++	±	++++
H. influenzae	+++	++++	+++	++++	+++	++++	+	++++	+	++++	+	++++	++	++++	+	++++

All the test strains grow well on this medium. The same result has been obtained when twice the quantity of the acidified and neutralized sheep blood is added to 15 cc. of agar.

Alkalinization.—In this experiment the process was reversed. Diluted sheep blood was alkalinized with N/1 NaOH and then neutralized with N/1 HCl. 0.5 cc. of this mixture was added to 15 cc. of agar and a plate poured. No growtb or very poor growth was obtained when the test organisms were streaked on this medium (Table VII).

TABLE VI

Thermolability of the Inhibitory Factor in Sheep and Cow Blood

Media: 0.3 per cent sheep blood agar with a constant amount of V.
0.3 per cent cow " " " " " " " "

Organisms	Sheep		Cow	
	56°C. for 1 hr.	68°C. for 30 min.	56°C. for 1 hr.	68°C. for 30 min.
B. parainfluenzae hemolyticus	±	++++	±	++++
H. hemolyticus (bacillus X)	±	++++	±	++++
H. influenzae	+	++++	+	++++

TABLE VII

Comparison of Growth on Sheep Blood Treated with Acid and Treated with Alkali

Media: 0.3 per cent acidified and neutralized sheep blood plate with a constant amount of V.
0.3 per cent alkalinized and neutralized sheep blood plate with a constant amount of V.

Organisms	Acidified and neutralized sheep blood	Alkalinized and neutralized sheep blood
B. parainfluenzae hemolyticus	+++	±
H. hemolyticus (bacillus X)	+++	±
H. influenzae	+++	+

These experiments seem to indicate that the inhibitory factor can be destroyed by acidification but not by alkalinization. It is of interest that Fildes (12) found trypsin digestion of sbeep or ox blood unsatisfactory as a means of improving the growth of *H. influenzae*. This author obtained good results by usiog peptic digestion of sheep or ox blood. In the case of peptic digestion the amount of acid (0.12 cc. pure HCl to 1 cc. of sheep blood) added by Fildes should be in itself sufficient to destroy the inhibitory action of sbeep blood, whereas with trypsin digestion an alkaline reaction is used.

9. Is the Inhibitory Factor Active in Fluid as Well as in Solid Media?—It seemed probable that if the failure of the influenza group of organisms to grow on sheep blood is due to an inhibitory factor, it should be possible to demonstrate the inhibitory action in fluid as well as in solid media. The basic fluid medium consisted of Difco dehydrated beef heart infusion broth (pH 7.5) to which a sufficient amount of yeast extract was added.

It has been found that the addition of washed laked sheep cells to broth inhibits the growth of the test organisms markedly, both aerobically and anaerobically. The reduction in growth has been confirmed by pour plates. No inhibitory action has been noted with intact washed sheep cells which tend to settle at the bottom of the tube. Large quantities of sheep serum added to broth have no inhibitory effect.

10. Nature of the Inhibitory Factor.—Adsorption with Charcoal.—2 gm. of bone charcoal were added to 5 cc. of laked sheep cells diluted 1:10 with distilled water and the mixture was incubated overnight at 37°C. Although the charcoal removes all the pigment from the diluted blood, the inhibitory action of the colorless fluid thus obtained is still marked.

Filtration through a Berkefeld Filter.—Sheep cells were laked by diluting 1:10 with distilled water. This solution was then filtered through a Berkefeld filter (N). Filtration failed to remove the pigment. The first and last portions filtered were tested separately. No difference has been noted between the two lots. The inhibitory factor is removed from both.

Symbiosis.—It was noted by Rivers (4) that symbiosis improved the growth of *H. influenzae* on human blood. On rabbit and cat blood on the other hand, on which *H. influenzae* grows well in pure culture, the size of the colony is not increased by the presence of other organisms. We have observed the same phenomenon with sheep blood: the activity of the inhibitory factor is reduced in the vicinity of accidental contaminants such as the staphylococcus.

11. Mechanism of the Inhibitory Action of Sheep Blood.—It was thought possible that the inhibitory action of sheep blood might be due to a destruction of the V factor by enzymes present in the erythrocytes. However, no definite experimental evidence has been obtained for this hypothesis.

DISCUSSION

Many types of selective media have been used to facilitate the isolation of various kinds of bacteria: aniline dyes for members of the typhoid-paratyphoid group, potassium tellurite for *Corynebacterium diphtheriae*, and sodium oleate for *H. influenzae*. The appearance of beta hemolytic streptococci on blood agar plates is usually so characteristic that no special selective medium has been considered necessary. When, however, the bacillus X (Pritchett and Stillman) or *B. parainfluenzae hemolyticus* (Rivers) are present in the throat

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cells through a Berkefeld filter removes the inhibitory factor without removing the pigment. These experiments seem to indicate that the inhibitory factor is not bound to the pigment of the erythrocyte.

It was thought of interest to examine the bloods of other animals to see how they compare with the rabbit and sheep. Blood was obtained from goats and cows, animals closely related to sheep, and from guinea pigs and rats for comparison with the rabbit. It has been found that goat and cow bloods inhibit the growth of the hemolytic bacilli and *H. influenzae* in the same way as sheep blood. No essential differences have been noted between the properties of the inhibitory factors in cow and goat blood and those described above for the inhibitor in sheep blood.

Unheated guinea pig and rat bloods are like rabbit blood in that they give good growth of *B. parainfluenzae*, *H. hemolyticus*, and *H. influenzae*. These organisms also grow moderately well on unheated horse blood. The growth of these bacilli on media containing 5 per cent human blood is poor and irregular. The inhibitor present in human red cells is, however, less powerful than that in sheep blood; it requires approximately ten times as much human blood to inhibit as it does sheep blood. The inhibitor in human blood, like that in sheep blood, is destroyed by heating at 68°C. for 30 minutes.

CONCLUSIONS

1. 5 per cent sheep blood agar is a selective medium for beta hemolytic streptococci in throat cultures since sheep blood inhibits the growth of bacillus X (*H. hemolyticus*) and *B. parainfluenzae hemolyticus*. The growth of *H. influenzae* is also inhibited by sheep blood.

2. The inhibitory action of sheep blood resides in the erythrocytes and is thermolabile. Disruption of the cell by laking has no effect upon the inhibitor.

3. The bloods of animals closely related to the sheep, such as the goat and the cow, have a similar inhibitory action on the growth of hemolytic and non-hemolytic members of the influenza group, while human blood contains a similar but less powerful inhibitor for these organisms.

4. Members of the influenza group grow well on unheated rodent blood: rabbit, guinea pig, and rat.

5. These organisms also grow fairly well on unheated horse blood.

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EXPERIMENTAL SYPHILIS OF ORIENTAL ORIGIN: CLINICAL REACTION IN THE RABBIT

By LOUISE PEARCE, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research, New York, and the Department of Medicine, Peiping Union Medical College, Peiping, China*)

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For many years it has been reported that the clinical manifestations of syphilis in tropical and oriental regions differ in several important respects from the disease usually seen in occidental countries. The most striking and frequently mentioned differences are a low incidence of general paresis and tabes, and the frequently severe character of secondary manifestations. Although the majority of reports lack laboratory evidence, the clinical observations are impressive, especially with respect to the comparative infrequency of parenchymatous neurosyphilis. Several suggestions have been advanced to account for this so called exotic syphilis. Among the first of these was the strain theory, that is, that strains of *Treponema pallidum* differ in their disease producing properties. Special or peculiar qualities of reaction of different races have also been invoked, and lately consideration has been given to the influence of recently introduced treatment and the presence of endemic malaria. It has also been suggested that, if, as some have thought, involvement of the central nervous system is a relatively late feature in the biological history of syphilis in the western world, one would not expect its incidence to be as high in communities in which the disease has recently been introduced as in those in which it has been present for many centuries.

The question of differences of syphilitic reaction as indicated by such terms as occidental, oriental, exotic and tropical syphilis has a larger significance than is ordinarily implied by such designations. An integral part of the subject concerns the essential nature of the tropical disease yaws or framhesia which some observers contend is in reality a form of syphilis. The precise status of another disease, "bejel," described as the endemic syphilis of the Euphrates Arab is still unde-

termined (1). This disease, which is non-venereally acquired, has little effect upon the general constitution, its lesions are principally mucocutaneous, cutaneous and periosteal, while the nervous and cardiovascular systems and the viscera escape serious damage. Syphilis in rural populations of southeastern Europe is also said to be endemic and relatively mild (2).

It has not been possible until comparatively recently to study the subject experimentally in the laboratory, but our knowledge of the biology of experimental syphilis in the rabbit is now sufficiently comprehensive to permit the investigation of certain features of the matter. To this end a study of two oriental strains of *T. pallidum* of Chinese origin was undertaken first in Peiping and subsequently in New York on the basis of the character of the reaction to infection in the rabbit as compared with two well known occidental strains of American origin. The results of these comparative experiments are reported in the present paper.

A special feature of the investigation concerned the animals employed. To avoid the complications which might be introduced by a variable animal material, rabbits of both American and Chinese sources were used in all experiments. The American rabbits were shipped from New York to Peiping shortly before the Peiping experiments and similarly Chinese rabbits were sent to New York. It is of interest to note that the animals stood the long voyage *via* the Panama Canal and the Yellow Sea, transhipment in Japan, and a rail journey in China remarkably well.

Materials and Methods

The study comprised four experiments, two in Peiping in 1931–32 and two in New York in 1932–33. Four strains of *T. pallidum* were employed, two of oriental and two of occidental origin. The oriental strains of *T. pallidum* employed, designated Chinese III and IV respectively, had been isolated 5 and 3 years previously from patients in the hospital of the Peiping Union Medical College. Both strains were known to produce well marked clinical manifestations in the rabbit. Chinese III strain was obtained by the intratesticular inoculation of rabbits of cerebrospinal fluid from a patient with an acute meningitis and a primary chancre. Dark field examination of the centrifuged fluid showed actively motile spirochetes. The strain was in the 12th generation of rabbit passage at the beginning of these experiments. Chinese IV strain was similarly obtained from an inguinal lymph node of a patient with secondary syphilis. There were multiple bone lesions and a

marked general glandular enlargement. The strain was in its 11th rabbit passage. The American strains employed were the well known Nichols and Zinsser-Hopkins organisms, which have been used for many years in the majority of studies on experimental syphilis in the United States. In both Peiping experiments all four strains were used. In the first New York experiment the Nichols and the Chinese III strains were used and in the second New York experiment the Nichols and the Chinese IV strains.

For source material the western strains were always carried in American and the eastern in Chinese rabbits. The emulsion for inoculation was prepared from the tissue of an acute orchitis with sterile salt solution. Dark field examination of the suspensions showed from 2 to 6 actively motile organisms per microscopic field. The dose employed was 0.2 cc. injected intratesticularly or intracutaneously.

The net total number of rabbits inoculated was 320 (Table I). Observations made on animals which died during the experiments are not included. The great majority of rabbits, namely 262, were inoculated in one testicle; 106 were injected with the Nichols, 25 with the Zinsser-Hopkins, 65 with the Chinese III and 66 with the Chinese IV strains respectively. A supplementary group in which the intradermal route was used was included in the second Peiping experiment (Table I). 58 rabbits were inoculated in the skin of the sheath, 13 rabbits with each of the American strains and 16 rabbits with each of the Chinese strains.

The rabbits were adult males derived from American and Chinese stocks. The American rabbits were hybrids with Dutch, English and Lilac crosses predominating; the Chinese rabbits were Albino mixtures and many were rather small delicate animals. Comparable numbers of animals from both sources were represented in each experiment. The intratesticular experiments in Peiping comprised 50 American and 51 Chinese rabbits and those in New York 72 American and 89 Chinese rabbits. For the intradermal experiment 28 American and 30 Chinese animals were used.

The rabbits for each set of experiments were assembled at the same time. When inoculated, those of the first Peiping and New York experiments were 6 to 7 months of age while those of the second Peiping and New York experiments were 8 to 9 months old. Each rabbit was caged separately from the time of assemblage throughout the periods of shipment and experiment. The diet in Peiping consisted of soy beans, cabbage and hay, and in New York of commercial food pellets, oats, hay and a free supply of water. These diets were also used during the two voyages.

The basis of comparison of the strains was the character of the clinical reaction to infection, and detailed clinical examinations were carried out at least three times a week. Special attention was paid to the following: (*a*) the frequency and time of occurrence of the primary orchitis; (*b*) the frequency and time of occurrence of critical edema in the inoculated testicle; (c) the frequency and time of occurrence of lesions in the uninoculated testicle (metastatic orchitis); (*d*) the frequency, time of occurrence and number of generalized lesions in the bones and periosteum, the

TABLE I

Experimental Data

Strain of *T. pallidum*	Experiment	Number and source of rabbits		
		Total	American	Chinese
Intratesticular inoculation				
Nichols	Peiping 1	10	5	5
	"	16	7	9
	New York 1	40	20	20
	" " 2	40	17	23
	Total....	106	49	57
Zinsser-Hopkins	Peiping 1	9	5	4
	" 2	16	8	8
	Total....	25	13	12
Chinese III	Peiping 1	9	5	4
	" 2	16	7	9
	New York 1	40	20	20
	Total....	65	32	33
Chinese IV	Peiping 1	9	5	4
	" 2	16	8	8
	New York 2	41	18	23
	Total....	66	31	35
	Total............	262	125	137
Intracutaneous inoculation				
Nichols	Peiping 2	13	5	8
Zinsser-Hopkins	" "	13	7	6
Chinese III	" "	16	8	8
Chinese IV	" "	16	8	8
	Total.............	58	28	30
Total.............	....	320	153	167

skin and mucous membranes, and the eye; (*e*) the proportion of animals that showed complete healing of all lesions at the termination of the experiments. The period of observation in the Peiping experiments was 4 months and in the New York experiments 3⅓ months.

In the analysis of the results group means have been employed, a procedure which tends to minimize the effect of the chance occurrence of severe syphilis in any one group. In the case of generalized lesions, the numbers given represent the numbers of discrete foci at which lesions developed as determined by actual count. The figures for actual rates or actual distribution represent the mean values for those animals of the group in which generalized lesions actually developed while the relative rates indicate the results in terms of the entire group.

RESULTS

The results of the experiments are recorded in Tables II to VII. The four principal experiments, two in Peiping and two in New York, in all of which the intratesticular route of inoculation was employed will first be discussed, and then follows the supplementary experiment in which the intradermal route of inoculation was used. The particular features of the infection selected for discussion are the outstanding clinical manifestations of the experimental disease.

Primary Orchitis.—Every rabbit of the 262 inoculated intratesticularly developed a primary orchitis (Table II). The mean incubation periods of the lesion were 18.5 and 14.5 days respectively for the Nichols and Zinsser-Hopkins strains and 18.9 and 15.8 days for the Chinese III and IV strains (Table III). It will be noted that for each strain there was comparatively little variation of the mean values of individual experiments from the mean group value. The greatest variation occurred with the Chinese III strain, the incubation period of the three experiments being 21.0, 18.4 and 17.3 days respectively.

Critical Edema.—Edema of the scrotum and tunics of the inoculated testicle is a less constant feature than other phenomena of infection and variability of results is to be expected. Its mean incidence in these experiments was as follows: Nichols strain 53.8 per cent; Zinsser-Hopkins strain 34.7 per cent; Chinese III strain 36.5 per cent, and Chinese IV strain 52.8 per cent (Table II). The values for the Nichols and the Chinese IV groups do not include the results of the second New York experiment because at the time when edema was appearing in these rabbits, rabbit pox (3) had developed in the colony. In certain instances it was thought that the edema was more probably related to pox than to the syphilitic process, and consequently the figures relating to this feature were omitted. There was considerable variation in edema incidence in the individual experiments. Considering only the two Peiping experiments the results with both Chinese strains were less variable than those with the American strains. In the first New York experiment the value for the Nichols strain returned to the level of the first Peiping experiment, while that for the Chinese III strain was also increased and exceeded considerably both Peiping figures.

The mean times of development of critical edema were generally comparable, that is, 27.5 and 24.4 days after inoculation for the occidental strains and 26.9

and 22.8 days for the oriental strains respectively (Table III). There was a slightly greater variation in the individual experimental values from their group means in the case of the Chinese strains, that is, 2 days more or less than the group mean as compared with 1 day for the American strains.

TABLE II

Incidence of Various Phenomena of Infection and Focal Distribution of Generalized Lesions

Strain	Experiment	Number of rabbits	Primary orchitis	Critical edema	Metastatic orchitis	Distribution of generalized lesions: Incidence	Focal distribution: Actual	Focal distribution: Relative
			per cent	*per cent*	*per cent*	*per cent*		
Nichols	Peiping 1	10	100.0	70.0	90.0	100.0	5.6	5.6
	" 2	16	100.0	31.3	81.3	87.5	5.3	4.7
	New York 1	40	100.0	60.0	75.0	70.0	5.5	3.9
	" " 2	40	100.0	*	92.0	75.0	9.2	6.9
	Total.........	106	100.0	53.8	84.8	83.1	6.4	5.3
Zinsser-Hopkins	Peiping 1	9	100.0	44.4	77.8	100.0	5.9	5.9
	" 2	16	100.0	25.0	100.0	93.8	4.8	4.5
	Total.........	25	100.0	34.7	88.9	96.9	5.4	5.2
Chinese III	Peiping 1	9	100.0	33.3	66.7	88.9	5.6	5.0
	" 2	16	100.0	31.3	75.0	87.6	4.4	3.8
	New York 1	40	100.0	45.0	72.5	80.0	7.0	5.6
	Total.........	65	100.0	36.5	71.4	85.5	5.7	4.8
Chinese IV	Peiping 1	9	100.0	55.6	88.9	100.0	6.3	6.3
	" 2	16	100.0	50.0	80.0	87.5	4.9	4.3
	New York 2	41	100.0	*	80.4	68.3	5.6	3.9
	Total.........	66	100.0	52.8	83.1	80.9	5.6	4.8

* Omitted because of intercurrent pox infection.

Metastatic Orchitis.—Clinical involvement of the uninoculated testicle was a frequent feature of the disease produced by all four strains (Table II). The mean incidence with the Nichols, the Zinsser-Hopkins and the Chinese IV strains was comparable, that is, 84.8, 88.9 and 83.1 per cent respectively; it was lower with the Chinese III strain, 71.4 per cent. There was comparatively little variation

in the values for individual experiments with the Chinese strains; for the four experiments with the Nichols strain the incidence ranged from 75.0 to 92.5 per cent and for the two Zinsser-Hopkins experiments, the values were 77.8 and 100.0 per cent.

TABLE III

Mean Time of Occurrence of Various Phenomena of Infection Estimated from the Date of Inoculation

Strain	Experiment	Number of rabbits	Primary orchitis	Critical edema	Metastatic orchitis	Generalized lesions: First lesion	Generalized lesions: Mean of all	Generalized lesions: Last lesion	Generalized lesions: Duration of active period
			days	*days*	*days*	*days*	*days*	*days*	*days*
Nichols	Peiping 1	10	19.4	28.6	54.1	57.9	66.8	74.1	20.4
	" 2	16	18.9	26.6	53.7	60.3	71.1	88.4	28.1
	New York 1	40	18.5	27.3	52.0	52.2	57.8	66.9	15.2
	" " 2	40	17.1	*	50.9	58.2	65.5	74.8	13.7
	Total........	106	18.5	27.5	52.4	57.2	65.3	76.1	19.1
Zinsser-Hopkins	Peiping 1	9	14.6	25.2	69.3	56.9	68.0	73.1	19.0
	" 2	16	14.3	23.5	44.2	64.8	72.3	92.8	29.0
	Total........	25	14.5	24.4	56.8	60.9	70.2	83.0	24.0
Chinese III	Peiping 1	9	21.0	29.0	56.5	59.0	67.2	78.5	20.0
	" 2	16	18.4	26.6	53.0	57.1	60.8	71.1	17.1
	New York 1	40	17.3	25.1	47.4	43.7	47.1	57.7	16.4
	Total........	65	18.9	26.9	52.3	53.3	58.4	69.1	17.8
Chinese IV	Peiping 1	9	16.7	24.8	48.9	51.6	60.5	71.8	20.8
	" 2	16	15.5	20.8	44.6	54.8	59.8	70.7	20.2
	New York 2	41	15.5	*	46.5	49.6	54.0	63.1	15.2
	Total........	66	15.8	22.8	46.7	52.0	58.1	68.5	18.7

* Omitted because of intercurrent pox infection.

The mean time at which metastatic orchitis was first detected was 52.4, 56.8 and 52.3 days after inoculation for the Nichols, Zinsser-Hopkins and Chinese III strains respectively; it was definitely earlier, 46.7 days, for the Chinese IV strain (Table III). For the four individual experiments with the Nichols strain and the three with the Chinese IV strain, the values were quite uniform, the range of variation being only 2 days from the group mean. For the three experiments

with the Chinese III strain, however, this variation amounted to 5 days and with the two Zinsser-Hopkins experiments 13 days.

Generalized Lesions.—The most significant single index of pathogenicity of a strain of *T. pallidum* is the production of generalized lesions in remote parts of the body. This phase of the disease comprises lesions of the periosteum and bone, the skin and mucous membranes, and the eyes. Involvement of the scrotum and tunics is not usually included since these may result from the direct extension of the primary lesion.

The animal incidence of generalized lesions in these experiments was high and remarkably uniform, the mean values being 84.8 and 96.9 per cent respectively for the Nichols and Zinsser-Hopkins strains and 85.5 and 80.9 per cent for the Chinese III and IV strains (Table II). Considering only the Peiping experiments, the mean results were 93.8 and 96.9 per cent for the two American strains and 88.2 and 93.5 per cent for the Chinese III and IV strains. The values in New York were consistently lower than in Peiping. For the two Nichols groups the mean incidence was 72.5 per cent; for the single Chinese III and IV groups the values were 80.0 and 68.3 per cent respectively. The results for the two Zinsser-Hopkins and the three Chinese III experiments were quite uniform; for the four Nichols groups the incidence ranged from 70.0 to 100.0 per cent and for the three Chinese IV experiments from 68.3 to 100.0 per cent.

The focal distribution of generalized lesions was next considered. The mean values given in Table II show that the results for the four strains were of the same general order. The highest values for both actual and relative rates occurred with the Nichols strain, namely, 6.4 and 5.3 respectively, but the figures for the other strains are but slightly lower, that is, Zinsser-Hopkins 5.4 and 5.2; Chinese III 5.7 and 4.8; and Chinese IV 5.6 and 4.8. The grade of infection as judged by the values for individual groups was most severe with the Nichols strain in the second New York experiment; the values for the three other experiments with this strain were lower but very uniform. A similar uniformity is seen in the results of the experiments with the Zinsser-Hopkins strain and of the three with the Chinese IV strain. In the case of the three experiments with the Chinese III strain, the values were more irregular and the New York figures were slightly higher than those in Peiping.

The location of generalized lesions has been used as a further basis of comparison of the four strains of organisms (Table IV). With the Nichols strain the mean values and those of individual experiments as well showed a higher proportion of skin than bone lesions; with the Zinsser-Hopkins strain this relationship was reversed. The mean value for eye lesions was the same, 2.8 per cent, for both American strains. With the Chinese III strain, the mean values resembled those with the Hopkins strain as regards the bone-skin representation of lesions; and in two of the three individual experiments the results were of the same order. The proportion of eye lesions, however, was high, the mean value being 5.3 per cent. In the case of the Chinese IV strain the mean values for bone and skin lesions approximated those of the Nichols strain, that is, a larger proportion of skin le-

sions. In two of the three experiments, however, these values were almost identical. There was likewise a high proportion of eye lesions with the Chinese IV strain, the mean value being 6.8 per cent. Eye lesions developed in all three experiments with the Chinese IV strain, but they were not observed in one each of the experiments with the other strains.

The mean time of appearance of the first generalized lesion was slightly earlier with both Chinese than with the American strains, the mean values being 53.3 and 52.0 days after inoculation for the Chinese III and IV strains and 57.2 and

TABLE IV

Location of Generalized Lesions

Strain	Experiment	Total number	Bone		Skin		Eyes	
			No.	*per cent*	*No.*	*per cent*	*No.*	*per cent*
Nichols	Peiping 1	56	20	35.7	33	58.9	3	5.4
	" 2	75	32	42.7	40	53.3	3	4.0
	New York 1	154	65	42.2	89	57.8	0	—
	" " 2	276	34	12.3	238	86.2	4	1.5
	Mean........		38	33.2	100	64.0	3	2.8
Zinsser-Hopkins	Peiping 1	53	30	56.6	23	43.4	0	—
	" 2	72	36	50.0	32	44.4	4	5.6
	Mean........		33	53.3	28	43.9	2	2.8
Chinese III	Peiping 1	45	25	55.6	15	33.3	5	11.1
	" 2	61	26	42.6	32	52.5	3	4.9
	New York 1	224	127	56.7	97	43.3	0	—
	Mean........		59	51.6	48	43.1	3	5.3
Chinese IV	Peiping 1	57	27	47.4	26	45.6	4	7.0
	" 2	69	32	46.4	31	44.9	6	8.7
	New York 2	156	55	35.3	95	60.9	6	3.8
	Mean........		38	43.0	51	50.5	5	6.5

60.9 days for the Nichols and the Zinsser-Hopkins strains (Table III). The values for individual experiments were in general agreement with the mean group values with the exception of the Chinese III strain, the figures for which were 59.0, 57.1 and 43.7 days respectively. The mean time of appearance of the last generalized lesion was likewise earlier in the case of both Chinese strains, being 69.1 and 68.5 days for the Chinese III and IV strains and 76.5 and 83.0 days for the Nichols and Zinsser-Hopkins strains (Table III). A similar result occurred with respect to the mean time of development of all generalized lesions. For both these

phenomena, the results of individual experiments were in fair accord with their group means except in the case of the Chinese III strain which again showed considerable variation. The interval between the appearance of the first and the last generalized lesion has been selected to represent the period of generalized disease activity. The duration of this period was slightly but definitely shorter for both Chinese strains, the mean group values being 17.8 and 18.7 days for the Chinese III and IV strains and 19.1 and 24.0 days for the Nichols and the Zinsser-Hopkins strains. The mean duration periods of individual experiments showed more uniformity for the Chinese than the American strains.

Recovery.—At the end of each experiment all animals were classified on the basis of complete resolution and healing of all lesions (Table V). The observation period of the Peiping experiments was 4 months and of those in New York $3\frac{1}{3}$ months. The proportion of completely negative animals was much higher with the Chinese than the American strains in the Peiping experiments, the mean values being 85.4 and 64.6 per cent for the Chinese III and IV strains as compared with 10.0 and 21.2 per cent for the Nichols and Zinsser-Hopkins strains. In the first New York experiment none of the rabbits inoculated with the Nichols strain had completely recovered as contrasted with 8.1 per cent recoveries among the rabbits infected with the Chinese III strain. This latter value, however, might be raised to 21.6 per cent by the inclusion of 5 rabbits which were "almost or practically negative." By this term is meant a slight fibrous residual thickening at the site of a previous lesion, and in the great majority of cases the condition eventually disappears. In the second New York experiment with the Nichols and the Chinese III strain, the proportion of complete recoveries was the same for both strains, 17.5 per cent. If a similar inclusion of 5 and 8 practically negative rabbits be made, the resulting values are 25.0 per cent for the Nichols and 36.6 per cent for the Chinese IV strain. It seems clear, therefore, that the clinical manifestations were definitely more enduring in the infections produced by the Nichols and Zinsser-Hopkins strains of *pallidum* than by the two Chinese strains employed.

Intracutaneous Inoculation.—The second Peiping experiment included 58 rabbits inoculated intracutaneously, 13 with each of the American strains and 16 with each of the Chinese strains.[1] The injections were made in the skin of the sheath with the same tissue emulsions used for intratesticular inoculation.

A primary chancre developed in each rabbit (Table VI). The mean incubation periods for the Nichols and the Zinsser-Hopkins groups were 23.3 and 14.8 days and for the Chinese III and IV groups, 20.4 and 17.4 days respectively. In general

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[1] 16 rabbits were inoculated with each strain, but there were 3 early accidental deaths in the groups inoculated with the Nichols and the Zinsser-Hopkins strains.

the chancres produced by both western strains were larger, more indurated and more destructive than those of the Chinese strains; 75.0 per cent of the chancres in the former groups were classified as large or medium sized as compared with 50.0 per cent in the latter groups. Metastatic orchitis developed in approximately one-third of the rabbits inoculated with the Nichols and the two Chinese strains, but there was a higher incidence, 53.8 per cent, in the Zinsser-Hopkins group (Table VI). The mean incubation period of the orchitis was practically the same, 76 and 78.8 days for the Chinese strains; it was shorter, 67 days, for the Nichols strain, but for the Zinsser-Hopkins strain it was prolonged to 92.9 days.

TABLE V

Number and Percentage of Animals with Complete Healing of All Lesions at Termination of Observation Period. Peiping Experiments 4 Months; New York Experiments 3½ Months

Strain	Experiment	Number of rabbits	Complete healing of all lesions	
			No.	*per cent*
Nichols	Peiping 1	10	2	20.0
	" 2	16	0	—
	Total............	26		10.0
	New York 1	35*	0	—
	" " 2	40	7	17.5
Zinsser-Hopkins	Peiping 1	9	1	11.1
	" 2	16	5	31.3
	Total............	25		21.2
Chinese III	Peiping 1	9	7	77.1
	" 2	16	15	93.7
	Total............	25		85.4
	New York 1	37†	3	8.1
Chinese IV	Peiping 1	9	6	66.7
	" 2	16	10	62.5
	Total............	25		64.6
	New York 2	40‡	7	17.5

Final observations on 9 rabbits which died during the last fortnight of the experiments are not included in this summary: *5 rabbits; †3 rabbits; and ‡1 rabbit.

As was to be expected with this route of inoculation, the animal incidence of generalized lesions was lower and their number smaller than was found with the intratesticular route. For the Nichols and Zinsser-Hopkins strains, the mean incidence was 38.5 and 84.6 per cent and for the Chinese strains III and IV, 75.0 and 56.3 per cent (Table VI). The focal distribution rates of the four groups were comparable, the actual rates being 3.5 and 3.0 for the American strains and 2.5 and 3.8 for the Chinese III and IV strains respectively.

TABLE VI

Incidence and Mean Time of Occurrence of Various Phenomena of Infection and Focal Distribution of Generalized Lesions. Intracutaneous Inoculation

Strain	Number of rabbits	Primary chancre		Metastatic orchitis		Generalized lesions		
		Incidence	Incubation period	Incidence	Incubation period	Incidence	Focal distribution	
							Actual	Relative
		per cent	*days*	*per cent*	*days*	*per cent*		
Nichols	13	100.0	23.3	30.7	67.0	38.5	3.5	1.3
Zinsser-Hopkins	13	100.0	14.8	53.8	92.9	84.6	3.0	2.5
Chinese III	16	100.0	20.4	37.5	76.0	75.0	2.5	1.9
Chinese IV	16	100.0	17.4	31.5	78.8	56.3	3.8	2.1

TABLE VII

Location and Mean Time of Occurrence of Generalized Lesions from the Date of Inoculation. Incidence of Complete Healing of All Lesions at Termination of 4 Months Observation Period. Intracutaneous Inoculation

Strain	Number of rabbits	Generalized lesions									Duration of generalized lesion activity	Animal incidence of complete healing of all lesions	
		Total number	Bone		Skin		Eyes		First lesion	Last lesion			
			No.	*per cent*	No.	*per cent*	No.	*per cent*	*days*	*days*	*days*	No.	*per cent*
Nichols	13	17	3	17.6	14	82.4	0	—	81.8	96.0	24.5	3	23.1
Zinsser-Hopkins	13	33	7	21.2	25	75.8	1	3.0	88.7	103.9	32.5	4	30.8
Chinese III	16	30	13	43.3	16	53.3	1	3.3	60.5	79.7	23.6	12	75.0
Chinese IV	16	34	6	17.6	24	70.6	4	11.7	84.9	97.7	18.6	12	75.0

The mean time of appearance of the first generalized lesion was approximately the same for the two American and the Chinese IV strains, the values being 81.8,

88.7 and 84.9 days after inoculation; for the Chinese III strain, however, it was considerably shorter, that is, 60.5 days (Table VII). A similar result occurred with respect to the appearance of the last lesions, that is, 96.0, 103.9 and 97.7 days for the two American and the Chinese IV strains and 79.7 days for the Chinese III strain. The mean duration of generalized disease activity for the American strains was 24.5 and 32.5 days; it was slightly shorter, 23.6 days, for the Chinese III strain and considerably shorter, 18.6 days, for the Chinese IV strain.

As is the rule with the intracutaneous route of inoculation, there was a predominance of skin and mucous membrane lesions (Table VII). The proportion of these lesions was 82.4, 75.8 and 70.6 per cent for the Nichols, Zinsser-Hopkins and Chinese IV strains respectively; a lower value, 53.3 per cent, occurred with the Chinese III strain. Involvement of the eyes was frequent with the Chinese IV strain, the proportion of lesions being 11.7 per cent as compared with 3.0 and 3.3 per cent with the Zinsser-Hopkins and Chinese III strains. No lesions of the eyes developed in the rabbits inoculated with the Nichols strain.

At the conclusion of the experiment, 4 months after inoculation, it was found that the incidence of rabbits in which all lesions had completely regressed and were healed was much higher in the animals inoculated with the Chinese strains. The actual values were 75.0 per cent for each Chinese strain and 23.1 and 30.8 per cent for the Nichols and Zinsser-Hopkins strains respectively (Table VII).

DISCUSSION

Before discussing the results of these experiments, one matter affecting the interpretation of the New York experiments should be referred to. In both these experiments there was an intercurrent rabbit pox infection and the question arises whether the findings can properly be compared with those previously obtained in Peiping.

The fact is well recognized that the manifestations of two concomitant infections may not be of the same order as those of each infection when present alone. It has been shown, moreover, that, under certain conditions and particularly those associated with the relative times of inoculation, the manifestations of experimental syphilis of the rabbit are affected by a concomitant vaccinal infection (4). In the present instance the outbreak of pox occurred toward the end of the first New York experiment, that is, at the beginning of the 4th month after inoculation. The experiment was at once terminated and no lesion not known to have been present before pox was detected was included in the results. The second New York experiment was well under way before the pox outbreak. It occurred just prior to the development of the syphilitic critical edema. Since scrotal edema also occurs in pox, the observations on this feature of the results were omitted from present consideration. That the intercurrent infection had some effect upon the subsequent course of the syphilitic condition is not improb-

able. For example, the comparatively short duration of generalized disease activity in this experiment (Table III) may be an instance in point. It will be noted, however, that the findings of all experiments have been presented separately and comparison of the tabulated values shows that the majority of results of the second New York experiment did not disagree in any major particular with those obtained in the previous experiments. It will also be noted that the evaluation of the final condition of the animals with respect to the proportion of rabbits which showed complete resolution and healing of lesions was based primarily on the longer and uncomplicated Peiping experiments.

The results of the four experiments here reported show first, that the general grade of infection produced in the rabbit by two strains of *T. pallidum* of Chinese origin was quite similar to that produced by two occidental strains, the Nichols and the Zinsser-Hopkins. There were no noteworthy differences in the incidence of primary and metastatic lesions nor in the severity of the generalized phase of the disease as judged by the focal distribution rate of generalized lesions. In pathogenicity both Chinese strains were comparable to the high level of the American strains. This result recalls the similar experience of Hu (5) with another and recently isolated Chinese strain, the pathogenicity of which was likewise pronounced from the first rabbit passage.

Although the oriental strains did not appear to evoke any quantitative peculiarities of reaction, there were certain qualitative differences between the disease produced by them and the American strains. The most evident difference concerned involvement of the eyes. Of the total number of generalized manifestations the mean proportion of eye lesions was 2.8 per cent for each American strain as compared with 5.3 and 6.5 per cent for the Chinese III and IV strains respectively (Table IV).

In the longer and uncomplicated Peiping experiments the animal incidence of eye lesions was 2.8 per cent for each Chinese strain and 2.3 and 0.8 per cent for the Nichols and Zinsser-Hopkins strains. The incidence of eye lesions in terms of their total possible number was: Chinese III strain 16.0 per cent; Chinese IV strain 16.0 per cent; Nichols strain 11.5 per cent; and Zinsser-Hopkins strain 8.0 per cent. Finally, of the generalized lesions, the mean proportionate values for eye lesions were 4.7 and 2.8 per cent for the Nichols and Zinsser-Hopkins strains as compared with 8.0 and 7.0 per cent for the Chinese III and IV strains respectively.

Eye lesions were not observed in the first New York experiment. In the sec-

ond, however, their proportionate values were 3.8 and 1.5 per cent for the Chinese IV and Nichols strains respectively (Table IV). The results of individual experiments, furthermore, showed generally higher proportionate values for the Chinese strains, the mean values for the American strains ranging from 1.5 to 5.6 per cent as compared with 3.8 to 11.1 per cent for the Chinese strains (Table IV).

Second, the infections produced by the Chinese strains and particularly the IV strain, showed a tendency toward slightly shorter incubation periods for the later phases of reaction (Table III). The mean incubation period of the metastatic orchitis was 46.7 days after inoculation for the Chinese IV strain, 6 days earlier than that of the Nichols and Chinese III strains and 10 days earlier than that of the Zinsser-Hopkins strain. The mean time of appearance of the first generalized lesion was 53.3 and 52.0 days after inoculation for the Chinese III and IV strains as compared with 57.2 and 60.9 days for the Nichols and Zinsser-Hopkins strains respectively. Similar differences occurred with respect to the mean time of appearance of the last generalized lesion and of all lesions. The difference for the duration of generalized disease activity is somewhat less marked as far as total mean values are concerned, but considering only the Peiping experiments, the mean values were 24.3 and 24.0 days for the Nichols and Zinsser-Hopkins strains and 18.6 and 20.5 days for the Chinese III and IV strains respectively.

Third, the tendency toward regression and healing of lesions was much greater with both Chinese strains. In the longer Peiping experiments the proportion of rabbits which were clinically negative at the conclusion of the experiments was 85.4 and 64.6 per cent for the Chinese III and IV strains as compared with 10.0 and 21.2 per cent for the Nichols and Zinsser-Hopkins strains respectively (Table V). The findings of the shorter and complicated New York experiments showed a similar tendency. It is probable that this acceleration of the latent phase was related to the shorter time periods of the later phases of the disease discussed above.

The results of the supplementary experiment in which the intradermal route of inoculation was used were in general agreement with those observed in the major part of the study in which the intratesticular route of inoculation was employed. The mean proportionate value of eye lesions was 3.3 and 11.7 per cent for the Chinese

III and IV strains as compared with 0.0 and 3.0 per cent for the Nichols and the Zinsser-Hopkins strains (Table VII). The mean time values for generalized disease activity tended to be shorter for the Chinese strains and the proportion of negative or healed rabbits at the end of the experiment was much higher (Table VII). In addition there was a general tendency for the primary chancres produced by the Chinese strains to be somewhat smaller, less destructive and less enduring than those produced by the American strains.

The predominant type of generalized manifestation with the Nichols strain (intratesticular inoculation) was involvement of the skin and mucous membranes. In three experiments slightly more than half and in the last experiment more than three-fourths the lesions were of this type (Table IV). In the two experiments with the Zinsser-Hopkins strain skin lesions represented slightly less than half the generalized manifestations and bone lesions were proportionately increased. The results for the Chinese strains were somewhat more variable but in general the Chinese III strain appeared to resemble the Zinsser-Hopkins strain with a tendency toward a higher proportion of bone than skin lesions. In two of the Chinese IV experiments bone and skin lesions were equally represented while in the third skin lesions greatly outnumbered those of the bones. But as has been said, eye lesions were a more frequent manifestation with both Chinese than with the American strains. This finding recalls the early experimental work of Nichols (6) and of Reasoner (7) in which involvement of the eyes was prone to occur with certain strains.

The predominance of one class of lesions over another seems to be primarily concerned with the immune reaction developed by the host. In an infection of average severity, the occurrence of well marked bone manifestations is apt to be associated with relatively few cutaneous lesions, while the development of numerous skin lesions is usually found in those animals with minor bone involvement. With the same strain of *pallidum*, considerable variation in the proportions of bone and skin lesions may be observed in individual rabbits of the same experiment and also in groups of rabbits. The liability of the eye to involvement is greater than that of most tissues since it is not protected to an equal extent by the general reaction that takes place in other parts of the body (8). It has been observed that these

lesions usually occur in advanced infections and are not commonly seen in animals that show a prompt and vigorous reaction except in cases in which the disease proves to be unusually severe. Both mild and severe types of reaction were represented in these experiments and no division of them on the basis of strains could be made. On the whole, the general level of disease severity in all the experiments was high, and it was remarkably constant for all four strains as indicated by the mean focal distribution rates for generalized lesions (Table II). In the present instance of the association of a comparatively high incidence of eye involvement with strains of *pallidum* of oriental origin, it is of interest to recall that lesions of the eyes in experimental syphilis bear some analogy to neurosyphilis of man on account of the circumstances surrounding their development.

A great deal of evidence has now been accumulated to show that various types of disease may be obtained by modifying the conditions under which the syphilitic infection is initiated. For example, such factors as the viability of the organisms, the route of inoculation, the sex and breed of rabbits employed, have a definite influence on the character of the disease. It is also affected by the season of the year, being always comparatively mild during the summer months while the periods of greatest severity are spring and fall. The pathogenic properties of a strain can also be modified to an extreme degree by simply varying the conditions of passage. Thus, the reaction of consecutive groups of rabbits inoculated intratesticularly with the Nichols strain by means of lymph node material obtained from rabbits similarly inoculated but which had long been clinically negative (late latency) was characterized by insignificant primary lesions and an almost total absence of generalized lesions (9).

It is clear that the disease picture is not fixed and predictable, but is rather a variable quantity, and that many conditions may favor or prevent the occurrence of lesions of any given class. Little is known of such conditions, of the nature of their operation or of the extent to which they may function, but the fact that they apparently act most frequently through the medium of the host does not rule out the possibility that the invading organism may also be affected.

The present experiments were designed to control as many variables as possible and thus to take into account the effect of possible modify-

-ing factors. Large numbers of rabbits of various stocks were used and four experiments were carried out in two widely separated laboratories over a 2 year period. The results show that in most respects the infections produced by two Chinese strains of *T. pallidum* were entirely comparable to those produced by the highly pathogenic Nichols and Zinsser-Hopkins strains of American origin. Nevertheless, a higher incidence of eye lesions and an acceleration of certain time relations of the reaction, notably the production of a higher proportion of latent cases within the time limits of the experimental period, were observed with the Chinese strains.

It is obvious that the reactions to the Chinese and American strains were not wholly identical. To attribute to the Chinese strains a selective affinity for the eye or a particular capacity to induce early latency is to ignore what is known of the biology of syphilitic infections with its marked tendency toward variation. It is unlikely that the strains in question possess special pathogenic properties of an inherent and fixed nature by virtue of which the variable effects were induced. It is much more likely that these results were referable to some peculiarity in the reaction of the animals which these organisms aroused. That the same variation of clinical picture would be encountered again under quite different circumstances or that the same strains would fail to reproduce this variation is entirely possible.

SUMMARY AND CONCLUSIONS

Two oriental strains of *T. pallidum* of Chinese origin were compared with two well known occidental strains, the Nichols and the Zinsser-Hopkins, on the basis of the clinical reaction to infection in the rabbit. Four experiments were carried out, two each in Peiping and New York. Animals from American and Chinese sources were represented in each experiment.

The results obtained showed that the reaction to both Chinese strains was generally comparable to that of the American strains. But with both Chinese strains, lesions of the eyes were more frequent, the time relations of certain phases of the reaction were shortened, and there was a higher incidence of complete recovery (latency) within the observation period. In view of the well known variability of the syphilitic reaction, however, it was not felt that these qualitative

differences in clinical response could be ascribed to inherent or biologically fixed properties of the strains.

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HEPATIC DYSFUNCTION IN DOGS FED DIETS CAUSATIVE OF BLACK TONGUE

By C. P. RHOADS, M.D., and D. K. MILLER, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research*)

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The morphologic alterations of tissue which result from a deficiency of the thermostable fraction of the vitamin B complex have been minutely described, but little information is available concerning those disorders of function which are present during life as indicated by the postmortem appearance. Gross as well as microscopic changes of the hepatic parenchyma are frequent in canine black tongue, a condition generally assumed to be the result of a deficiency of vitamin B_2 (G), and the disease can be prevented or cured by liver extract. No evidence has been advanced, however, that the histologic changes are associated with a derangement of hepatic function, even though they may be prevented or cured by the administration of hepatic substance. Accordingly a study has been made of the ability of the liver to excrete intravenously injected bilirubin, as affected by feeding a diet lacking in vitamin B_2 (G) and causative of canine black tongue.

The rate of excretion of injected bilirubin as a test of liver function was first described by von Bergmann (1) and by his associate, Eilbott (2). The study was taken up by Harrop and Barron (3) and confirmed. The test was applied by these workers to pernicious anemia, and it was found that liver dysfunction, by the bilirubin test, was a uniform feature of that disease. Soffer (4) applied the test in a study of liver function during pregnancy, and was satisfied that it was a delicate method of detecting early hepatic insufficiency. Strauss and Castle (5) have published evidence that vitamin B_2 (G) (the factor which is preventive and curative of canine black tongue) is similar to the extrinsic antipernicious anemia factor in its distribution and resistance to heat. Hence it was desirable, in studying hepatic function during the feeding of diets causative of black tongue, to apply a test which had been shown to demonstrate hepatic dysfunction in pernicious anemia. Therefore the quantitative measurement of the ability of the liver to excrete intravenously injected bilirubin was employed, after suitable modification which allowed it to be applied to the dog.

Histologic changes in the livers of dogs dying of acute black tongue have been

described by Lillie (6). In certain animals fed a diet causative of black tongue, and with the stomatitis which is characteristic of that condition, Sebrell (7) has described a "yellow liver" associated with a very marked fatty infiltration. This pathologic change, as well as the less striking but relatively constant hepatic lesions of uncomplicated black tongue can be prevented by the addition of dried or autoclaved yeast to the diet. Goldberger (8) showed that black tongue could be prevented and cured by supplementing the diet with the liver extract, which is effective in the treatment of pernicious anemia.

Methods

Crystalline bilirubin was obtained from the Eastman Kodak Company. Experiment showed that the dog has a much greater power to excrete injected bilirubin than does man, a rate of about 2 mg. per kilo per hour being common. In man the excretion of 1 mg. per kilo in 4 hours is considered normal.

2 mg. of bilirubin per kilo of body weight were dissolved in 15 cc. of 0.1 molar solution of sodium carbonate which previously had been brought to the boiling point and then allowed to cool to 80°C. A control sample of oxalated blood was taken; the pigment was injected and after an interval of 5 minutes, to allow thorough mixing, a second sample of blood was drawn. A third sample was taken 1 hour after injection. Perfectly dry syringes and needles were used to avoid hemolysis. The amount of bilirubin in the plasma was determined by the method of Ernst and Förster (9). The plasma was precipitated by acetone, using different concentrations according to the amount of bilirubin in the sample. After thorough shaking the mixture of acetone and plasma was centrifuged and read against the bichromate standards.[1]

The bilirubin content of the specimen taken 5 minutes after the injection, less the content of the first or control sample, was considered as containing 100 per cent of the injected pigment. The percentage of bilirubin retained in the third, or 1 hour plasma, was thus calculated after subtraction of the control figure.

The dogs were healthy mongrel animals averaging about 8 kilos in weight. The diet fed was that first described by Goldberger and used in many previous experiments (10) in this laboratory. It is known to produce acute black tongue after 5 to 12 weeks of feeding. Check tests of the ability of the animals to excrete intravenously injected bilirubin were made before the diet was fed and a normal figure was established for each animal. During the period of diet feeding the tests were repeated at intervals. The effect on the rate of excretion of bilirubin which resulted from supplementing the diet with 10 gm. of liver extract daily and from feeding the normal diet once more was tried. The liver extract was made up to a 25 per cent solution in water and was fed by stomach tube.

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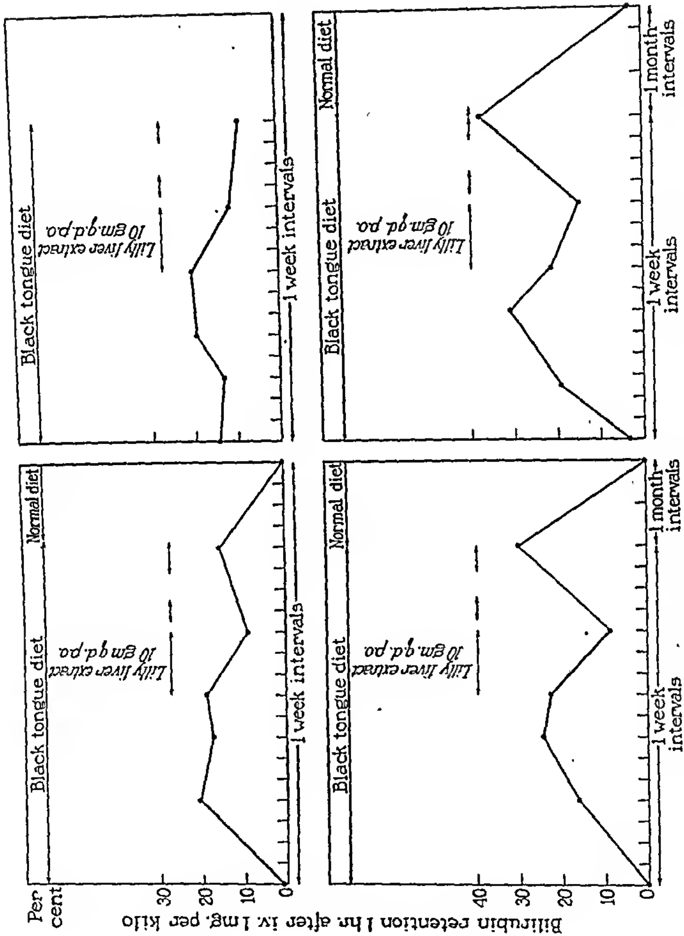
[1] Supplied by the La Motte Chemical Company.



TEXT-FIG. 1. The change in the rate of bilirubin excretion in dogs fed a deficient diet.

RESULTS

Four dogs were tested repeatedly to determine their power to excrete bilirubin under normal conditions, Text-fig. 1. In three there was no retention greater than 5 per cent. In the fourth animal the normal retention was between 12 and 15 per cent. In the first three instances the next test, done 4 weeks after the diet feeding was begun, showed a well defined retention ranging from 18 to 30 per cent. Test animal 4, with a normally low excretory function, showed no increase in retention at this period. At the second test done at the 5 to 7 week period of diet feeding, retention was present in all four animals and increased in three. No evidence of black tongue was present but all were losing weight.

Having established the fact of a progressive decrease in liver function during the diet feeding, the effect of supplementing the diet with 10 gm. daily of liver extract (Lilly N.N.R.) was studied. In all four animals an increased but still somewhat subnormal excretory power for bilirubin resulted.

The diet was then changed to a mixture of cooked meat, bread, and dog biscuit which is known empirically to maintain dogs in good health over a period of years. In every instance the excretory power for bilirubin was restored to normal by this move.

DISCUSSION

The results are clear cut in all four animals tested, and indicate a progressive decrease in the excretory power of the liver during the feeding of the deficient diet. Improvement in function followed the addition of liver extract, a finding in accord with the fact that liver extract is preventive and curative of canine black tongue. The incomplete restoration of normal hepatic function following the supplement with liver extract suggests that the diet is deficient in more than one factor, both of which are required for normal liver function. This is in accord with the findings of Ruffin and Smith (11) who showed that liver extract was not completely preventive of the Goldberger diet effect. The defect could be remedied in our experiments by feeding a normal mixed diet, good evidence that permanent functional damage had not been caused.

The exact nature of the lacking factor in the Goldberger diet has

not been ascertained. The recent studies of Street (12) indicate that black tongue may be caused by feeding synthetic diets lacking the heat-stable vitamin B complex other than flavin. Furthermore Koehn and Elvehjem (13) have shown that black tongue is not prevented or cured by supplementing the Goldberger diet with flavin (B_2), but that cure is effected by a substance in the B_2 complex which is not adsorbed by Fuller's earth or by alcohol and ether. They consider it similar to, if not identical with, the antidermatitis factor for the chick (filtrate factor). More accurate studies must await the identification of this substance.

CONCLUSIONS

1. The feeding to dogs of a diet lacking the vitamin B_2 (G) complex results in a lowered capacity of the liver to excrete intravenously injected bilirubin.

2. Normal function can be partly restored by supplementing the diet with liver extract.

3. Normal function can be completely restored by feeding a normal diet.

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THE ABSENCE FROM THE URINE OF PERNICIOUS ANEMIA PATIENTS OF A MOSQUITO GROWTH FACTOR PRESENT IN NORMAL URINE

By W. TRAGER, Ph.D., D. K. MILLER, M.D., and C. P. RHOADS, M.D.

(From the Department of Animal and Plant Pathology, The Rockefeller Institute for Medicol Research, Princeton, New Jersey, and the Hospital of The Rockefeller Institute for Medical Research, New York)

(Received for publication, November 30, 1937)

The larvae of the mosquito, *Aëdes aegypti,* require for their normal growth and development certain accessory growth substances which they obtain, in nature, from living microorganisms. All attempts to grow the larvae in the absence of living microorganisms proved unsuccessful, until it was found (1) that a medium containing heat-killed yeast and 0.5 per cent Lilly liver extract No. 343 (a partially purified preparation used for the treatment of pernicious anemia) would support normal growth under sterile conditions. The growth factor, designated as factor A, which is present in liver extract, cannot be supplied (2) by the highly purified anti-anemic preparations of Dakin and West (3) and Jacobson and Subbarow (4), showing that factor A and the antipernicious anemia principle are not identical. Factor A does, however, resemble the anti-anemic substance in the following ways. It is abundant in liver and kidney and less so in heart, while body muscle contains very little (2, 5). It can be adsorbed by charcoal and dialyzed through a collodion membrane (2, 6). It is heat-stable in neutral or slightly acid solution, but is destroyed by ashing, by boiling for 1 hour with 0.5 N sulfuric acid, or by exposure at room temperature for 24 hours to 0.5 N sodium hydroxide (2, 3). It is present in small amount in that portion of aqueous liver extract precipitated by 70 per cent alcohol, and in large amount in the precipitate obtained when the 70 per cent alcoholic filtrate is brought to a concentration of 95 per cent alcohol (2, 7).

These resemblances seemed to warrant further investigation. Wakerlin's finding (8) that normal human urine contains a substance

effective against pernicious anemia suggested the assay for mosquito growth factor of urine extracts from normal persons and from patients with aplastic anemia and with pernicious anemia before and after adequate treatment.

Two of us prepared the urine extracts, while the other performed the mosquito growth tests, usually in ignorance of the type of person from whom the extract was obtained.

Methods

1. Preparation of the Urine Extracts.—All the urine passed during a 24 hour period was collected with a few drops of toluene as a preservative and stored in a refrigerator. It was then measured, evaporated under reduced pressure at 55°C. to a volume of about 150 cc., and poured into a volume of 95 per cent alcohol sufficient to give a final concentration of 70 per cent alcohol. The mixture was allowed to stand overnight and was then filtered. The filtrate was concentrated under reduced pressure to about 100 cc. and was poured into enough absolute alcohol to give a concentration of 95 per cent alcohol. After vigorous shaking, a fine flocculent precipitate settled out. This was filtered off and dissolved in 100 cc. of distilled water. The pH was adjusted to 6.0 and the material was autoclaved ½ hour at 120°C.

2. The Mosquito Growth Test.—Significant results can be obtained only if the larvae are reared in the absence of living microorganisms. As in previous work on the nutrition of mosquito larvae (2), 1 to 2 day old eggs of *Aëdes aegypti* were sterilized on the outside and placed in tubes of sterile 0.5 per cent Lilly liver extract No. 343. 4 days later the young larvae were washed in sterile distilled water and inoculated into the experimental tubes. These were prepared by making suitable dilutions of the urine extract with sterile distilled water. Each tube contained a total of 6 cc. of medium and received 0.3 cc. of washed killed yeast suspension. Three larvae were inoculated into each tube. Each urine extract was tried in at least four different dilutions, using at least two tubes for each dilution. Some of the extracts were tested several different times with concordant results. The tubes containing the larvae were held in an incubator at 28° ± 1°C. and were observed daily at first and then every other day for a period of about 20 days, the number of larvae in each instar being noted.

RESULTS

(*a*) *In Urine Extracts.*—

It has been shown (2) that in the presence of killed yeast (0.1 cc. per larva) and 0.5 per cent Lilly liver extract No. 343 (or other suitable source of growth factor A) (2) all the larvae reach the third instar on the 3rd day, and nearly all reach the fourth instar on the

TABLE I

The Growth of Aëdes aegypti Larvae in Urine Extracts

Extract*	Concentration (fraction of total volume)	Reaching 3rd stage in 20 days	Average time to reach 3rd stage	Reaching 4th stage	Rating
		per cent	*days*	*per cent*	
C:1	1/3	0	—	0	
	1/6	0	—	0	−
	1/12	0	—	0	
	1/20	17	13	0	
C:2*a*	1/3	Toxic	—	0	
	1/6	33	8	0	+++
	1/12	33	9	0	
	1/20	17	6	0	
C:2*b*	1/6	33	9.5	0	
	1/12	33	7	0	++++
	1/20	66	9	0	
	1/60	17	11	0	
G:1	1/3	17	9	0	
	1/6	17	6	0	+
	1/12	17	6	0	
	1/20	0	—	0	
G:2	1/3	0	—	0	
	1/6	83	7	0	+++++
	1/12	67	5	33	
	1/20	50	8	0	
A:1	1/3	0	—	0	
	1/6	0	—	0	−
	1/12	0	—	0	
	1/20	17	11	0	
A:2	1/6	Toxic	—	0	
	1/12	17	9	0	+
	1/20	17	4	0	
	1/60	0	—	0	
A:3	1/3	83	6	0	
	1/6	67	7	0	
	1/12	33	5	0	++++
	1/20	0	—	0	
	1/60	17	6	0	

* The capital letter refers to the patient, the number to the extract, and the small letter to the trial as described in Table II.

TABLE II

Mosquito Growth Test of Various Urine Extracts

Patient	Description of case	Urine extract	Mosquito growth test
A	Housewife of 61. Paresthesia of extremities, 2 yrs. Gastrointestinal disturbance and anemia, 1 yr. Diminished perception of vibratory sense in the extremities. Erythrocytes 4,720,000; hemoglobin 100%; leukocytes 3,700. No free HCl in gastric juice. Treated twice weekly by intramuscular injection of liver extract (Lederle Laboratories) 1 cc. Full remission effected	1. Before treatment 2. Before treatment 3. After treatment	– + ++++
B	Housewife of 50. Pallor and weakness, 3 yrs. Paresthesia and difficulty in walking, 8 mos. Inadequate therapy had been given. Pallor, lingual atrophy, icteroid sclerae, and absent vibratory sense of the extremities. Erythrocytes 3,900,000; hemoglobin 87%; leukocytes 4,950; mean corpuscular volume 89. No free HCl in the gastric juice. Treated twice weekly with intramuscular injection of 10 cc. liver extract (Eli Lilly and Co.). Complete disappearance of symptoms	1. Before treatment 2. Before treatment 3. Before treatment 4. After treatment	– – + +++
C	Housewife of 39. Weakness and loss of weight, 1 yr. Diagnosis of pernicious anemia. Sore tongue and mouth. Paresthesia of extremities for 2 mos. Erythrocytes 2,080,000; hemoglobin 58%; leukocytes 5,000; mean corpuscular volume 121. No free HCl in gastric juice. Treated every 2 wks. with 10 cc. liver extract (Lilly) intramuscularly. Full remission effected	1. Before treatment 2. After treatment, trial *a* 2. After treatment, trial *b*	– +++ ++++

TABLE II—*Continued*

Patient	Description of case	Urine extract	Mosquito growth test
D	Married clerk of 48. Pallor and weakness, 6 yrs. Diagnosis of pernicious anemia for 5 yrs. with inadequate oral therapy. Paresthesia of extremities, 2 wks. Icteroid sclerae, atrophic glossitis, and diminished vibratory sense. Erythrocytes 1,380,000; hemoglobin 42%; leukocytes 3,100; mean corpuscular volume 140. No free HCl in gastric juice. Treated twice weekly with 10 cc. liver extract (Lilly) intramuscularly. Full remission effected	1. Before treatment, trial *a* 1. Before treatment, trial *b* 2. After treatment	+ ++ +++++
E	Italian-born male cigar worker of 54. Weakness and loss of weight, 2½ yrs. Oral liver extract, 1 yr. Paresthesia and loss of coordination, 1 yr. Marked loss of perception of vibration and position. Erythrocytes 2,490,000; hemoglobin 76%; leukocytes 4,400; mean corpuscular volume 133. No free HCl in gastric juice. Treated twice weekly with concentrated liver extract (Lederle) intramuscularly. Full remission effected	1. Before treatment 2. Before treatment 3. After treatment 4. After treatment	− − ++ +
F	Single seamstress of 55. Pallor and weakness, 5 yrs. Diagnosis of pernicious anemia for 4 yrs. with inadequate treatment. Paresthesia for 4 mos. Icteroid sclerae, atrophic glossitis, and diminished vibratory sense. Erythrocytes 1,400,000; hemoglobin 50%; leukocytes 4,350; mean corpuscular volume 149. No free HCl in gastric juice. Treated twice weekly with 10 cc. liver extract (Lilly) intramuscularly. Full remission effected	1. Before treatment 2. After treatment	+ ++

TABLE II—*Continued*

Patient	Description of case	Urine extract	Mosquito growth test
G	Housewife of 65. Stomatitis, pallor, weakness, 8 mos. Paresthesia, 2 mos. 30 lbs. loss of weight. Atrophic glossitis, icteroid sclerae, and diminished vibratory sense of the extremities. Erythrocytes 710,000; hemoglobin 22%; leukocytes 1,950; mean corpuscular volume 129. No free HCl in gastric juice. Treated twice weekly with 10 cc. liver extract (Lilly) intramuscularly. Full remission effected	1. Before treatment 2. After treatment	+ +++++
H	Married stationary engineer of 64. Pallor and weakness, 2 yrs. Diagnosis of pernicious anemia with inadequate therapy. Icteroid sclerae. Vibratory sense absent in lower extremities. Erythrocytes 1,400,000; hemoglobin 40%; leukocytes 2,700; mean corpuscular volume 122. No free HCl in gastric juice. Treated twice weekly with 10 cc. liver extract (Lilly) injected intramuscularly. Full remission effected	1. Before treatment 2. After treatment	++ ++++
I	Married unemployed male of 40 with stomatitis, weakness, and pallor for 7 yrs. Inadequate oral treatment with liver extract. Icteroid sclerae and very slightly diminished vibratory sense in the extremities. Erythrocytes 2,600,000; hemoglobin 74%; leukocytes 6,650; mean corpuscular volume 112. No free HCl in gastric juice. Treated by ultraviolet light for 2 wks. with moderate improvement of the blood. Treated twice weekly with 10 cc. liver extract (Lilly) injected intramuscularly. Full remission effected	1. Before treatment 2. After ultraviolet treatment 3. After liver extract treatment	− ++ ++++

TABLE II—*Concluded*

Patient	Description of case	Urine extract	Mosquito growth test
J	Cancer of intestine	1	+
K	Ulcerative colitis	1	+
L	Aplastic anemia	1	+++
M	" "	1	+++++
N	" "	1	++++
O	" "	1	++++
P	" "	1	+++
Q	Probable aplastic anemia	1	++
R	" " "	1	++++
S	Leukemia	1	++
T	"	1	++++
U	Normal	1	+++
V	"	1	+++++
W	"	1	++++
	"	2	+++

4th day and emerge as adult mosquitoes on the 9th day. With the same amount of killed yeast in distilled water (or in various other media not containing factor A) the larvae never get beyond the second instar, and they eventually die in this stage.

Preliminary trials with normal urine extract showed that while many of the larvae reached the third instar, only a few reached the fourth and none emerged as adults. Thus normal urine extract either does not contain enough factor A to bring about normal growth, or else contains only some of the substances which are responsible for the factor A activity. Concentrations of urine extract higher than 40 per cent by volume were generally toxic, all the larvae being dead 1 day after inoculation. In the middle range of concentrations, depending on the urine extract used, the larvae either survived for a long time in the second instar or reached the third instar and then survived at this stage.

Accordingly, the percentage of larvae reaching the third instar within 20 days was taken as the chief criterion of growth. The other criteria were the average time to reach the third instar and the percentage reaching the fourth instar (very small even in the most favorable cases). On the basis of these criteria the urine extracts were rated with respect to their growth factor content as –, +, ++,

etc. Table I gives some of the actual data and illustrates the method of rating.

The results with the various urine extracts tested and rated in this manner are given in Table II.

TABLE III

Mosquito Growth Test of Urine Extracts in the Presence of the Calcium-Filtrate Fraction†*

Medium		$N \times \frac{1}{T}$
Calcium-filtrate fraction only		5.1
Calcium-filtrate + flavine-purine complex (100 cc. of solution has material from 50 gm. liver)		23.1
Calcium-filtrate + urine extract W:2‡	1/6	16.0
	1/12	15.0
	1/24	12.2
" " " " C:2	1/6	13.6
	1/12	15.8
	1/20	10.3
" " " " D:1	1/6	7.4
	1/12	11.5
	1/20	7.1
" " " " D:2	1/12	17.1
	1/20	15.4
	1/60	10.1
" " " " A:2	1/12	9.2
	1/20	0
	1/60	0

* Concentration of urine extract expressed as fraction of total volume.

† Concentration of calcium-filtrate fraction always such that 100 cc. of solution contains the material derived from 50 gm. of liver.

‡ See Table II for description.

(*b*) *In Urine Extracts Supplemented with Certain Liver Fractions.*—Work, as yet unpublished, has shown that the mosquito growth factor A consists of at least two components.[1] One of these was iso-

[1] The work was done in collaboration with Dr. Y. Subbarow of The Harvard Medical School.

lated as a flavine-purine complex (1.2 per cent flavine-phosphate). The other was present in a fraction (designated as calcium-filtrate fraction) derived from the material obtained from liver extract by adsorption on charcoal and elution with alcohol (6). In the presence of killed yeast, neither of these two fractions alone supported normal growth of the larvae. But both together, in a concentration such that 100 cc. of solution contained that amount of each which was derived from 50 gm. of liver, gave entirely normal growth and metamorphosis. The method employed for the quantitative assay of factor A has been previously described (2). A growth index is obtained as a number, $N \times \frac{1}{T}$, which is determined by the percentage of larvae reaching the fourth instar in 10 days and the average time required to reach the fourth instar. When growth proceeds at an optimum rate, the value of $N \times \frac{1}{T}$ is $100 \times \frac{1}{4}$ or 25. Five of the urine extracts were tested in the presence, first, of an optimum concentration of flavine-purine complex and second, of an optimum concentration of calcium-filtrate factor. Growth in all the urine extracts plus flavine-purine complex was the same as in the urine extract alone, so that $N \times \frac{1}{T}$ equalled zero. But normal urine extract plus calcium-filtrate fraction gave growth almost as good as in flavine-purine complex plus calcium-filtrate fraction. These results are shown in Table III. It is worth noting that in this test, as well as in the test shown in Table II, extracts C:2, rated as +++, and D:2, rated as +++++, gave growth as good as did the normal extract W:2, rated as +++, while the growth obtained with D:1 and A:2, both rated as +, was markedly less.

DISCUSSION

The data of Table II demonstrate that normal urine, as well as urine from persons with aplastic anemia or leukemia, contains a substance which, under the described conditions, will enable many *Aëdes aegypti* larvae to reach the third instar, and a few to reach the fourth instar. In the urine of nine pernicious anemia patients this substance was absent or present in much smaller amount. Following a full remission of symptoms produced by liver extract therapy the

urine from all of these patients showed an increased amount of the mosquito growth factor. In the urine of seven of the nine patients the amount of this substance was greatly increased, reaching or slightly exceeding that present in normal urine. Interestingly enough, the urines from a patient with cancer of the intestine and from one with ulcerative colitis also showed a low content of growth factor.

The data of Table III give some indication as to the nature of this growth substance. Normal urine extract, or extract from the urine of adequately treated pernicious anemia patients, can replace the flavine-purine complex which is necessary for the growth of the mosquito larvae. Extract from pernicious anemia patients who show symptoms cannot replace the flavine-purine complex, giving growth but little better than that obtained with the calcium-filtrate fraction alone. Neither normal nor pernicious anemia urine extracts can replace the calcium-filtrate fraction. Hence we can tentatively conclude that the flavine-purine complex, or some material endowed with its potentialities for mosquito development, is excreted in much smaller amount by pernicious anemia patients showing symptoms than by normal individuals, persons with aplastic anemia, or adequately treated pernicious anemia patients. Normal urine extract, since it does enable a few larvae to reach the fourth instar, must also contain very small amounts of substances having effects like those of the calcium-filtrate fraction. The data thus far obtained give no information concerning the presence or absence of these substances in pernicious anemia urine extracts.

Several workers have shown that appreciable amounts of free flavine are excreted in normal human urine (9–13). No work has yet been reported on the flavine excretion of pernicious anemia patients. There is sufficient evidence that riboflavine is neither the anti-anemic factor (14), the "extrinsic factor" (15) or the pellagra preventive factor (16, 17). Nevertheless, it is still possible that there is, in pernicious anemia, an upset in the flavine metabolism. In this connection, the work of Laszt and Verzár (18) on chronic iodoacetate poisoning of rats is of especial interest. These investigators found that rats fed on a complete diet containing suitable amounts of iodoacetate failed to grow, and developed steatorrhea, osteoporosis,

skin symptoms, a decided anemia, and great hypertrophy of the suprarenals. All the effects could be completely counteracted if the rats were fed flavine-phosphate, but not if they were fed lactoflavine, indicating that the poisoning interfered with the phosphorylation of lactoflavine, a reaction necessary in the formation of yellow enzyme (19).

Miller and Rhoads (20) have shown that the livers of swine fed a modified Goldberger diet are deficient in antipernicious anemia substance. They have also found (21) that guinea pigs kept on this diet lose weight rapidly and die in 2 to 3 weeks unless the diet is supplemented with adequate amounts of liver extract or vegex, when the animals remain in normal health. An extract from the liver of a swine on this diet, and three extracts from the livers of groups of guinea pigs on this diet, were found to contain very much less mosquito growth factor A than normal swine and guinea pig liver extracts respectively. Unfortunately, these deficient extracts were not tested in such a manner as to determine whether they were lacking in flavine-purine complex or in calcium-filtrate fraction or in both components of factor A.

SUMMARY

Extracts prepared from the urine of normal persons or patients with aplastic anemia or leukemia contain a substance, possibly flavine or a flavine compound, which under suitable conditions of test enhances the growth of larvae of the mosquito, *Aëdes aegypti*. This substance is lacking, or is present in much smaller amount, in extracts from the urine of pernicious anemia patients showing symptoms of the disease. Extracts from the urine of the same patients after adequate treatment contain as much of the substance as normal urine extracts.

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THE METABOLISM OF LUNG TISSUE IN EXPERIMENTAL LOBAR PNEUMOCOCCUS PNEUMONIA IN THE DOG*

By THEODORE E. FRIEDEMANN, Ph.D., and JAMES B. GRAESER, M.D.

(*From the Department of Medicine, The University of Chicago, Chicago*)

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One of the outstanding features of pneumococcus lobar pneumonia is the intensity and magnitude of the inflammatory reaction which persists for a week or more and which in most instances subsides without any apparent damage to the tissue. The inflammation profoundly alters the conditions under which the tissue must metabolize. The alveoli are filled with exudate which is composed principally of leucocytes and which usually contains large numbers of pneumococci. The change in the alveoli is from a completely aerobic to an almost completely anaerobic environment. Oxygen for tissue respiration, which formerly was obtained from alveolar air, now must be derived mainly from venous blood. The leucocytes and pneumococci (Friedemann, 1) metabolize at a very rapid rate. The blood which formerly provided nutrients for the alveolar cells must now provide adequately for a greatly increased number of rapidly metabolizing cells; also, if the tissue is to survive, metabolites and toxic substances must be removed.

To what extent do the severe conditions imposed on the lung affect the metabolism of the tissue? Does the blood adequately supply oxygen and the necessary nutrients; and are the metabolites efficiently removed? To answer these questions we have analyzed, first, the tissue at various stages in the disease, and secondly, the blood entering and leaving the consolidated as well as the uninfected lung.

EXPERIMENTAL

Experimental Pneumococcus Pneumonia.—This was produced in dogs by the method of Terrell, Robertson, and Coggeshall (2). A highly virulent Type I

* This study was aided by grants from the Bartlett Memorial Fund and the Douglas Smith Foundation for Medical Research of The University of Chicago.

organism (A_5) was used throughout. It was found impossible to produce constantly lesions confined to single lobes, due to the natural variability in the experimental disease. Therefore, in order to classify the lesions according to their respective stage of development x-ray exposures were made at least once a day, and, at death, representative sections were studied histologically.

Analysis of Tissues.—The animals were killed by a 220 volt electric current applied for a period of 7 seconds. The electrodes, which consisted of heavy clamps, were attached to the lip and hind leg after moistening with salt solution. The chest was then immediately opened. Blood was withdrawn from the heart and representative samples of lung tissue were simultaneously dropped into Dewar flasks which contained liquid nitrogen. With sufficient assistance we were able to carry out the sampling in a minimum of 45 seconds, with an average for all experiments of about 90 seconds, from the time of application of the current. Immediately following this the exudate was vitally stained by the technique of Kredel and Van Sant (3). Specimens were then taken for histological examination.

The frozen samples were crushed and prepared for analysis by the technique of Graeser, Ginsberg, and Friedemann (4). The following analytical methods were used: lactic acid, Friedemann and Graeser (5); glucose, Shaffer-Hartmann as modified by Shaffer and Somogyi (6); glycogen, Somogyi (7).

As a control on the technique, blood was taken from the saphenous vein of 6 of the animals before electrocution, and from the heart after death. The glucose, lactic acid, and non-protein nitrogen content of both samples agreed in all cases within the limits of experimental error of the methods. It would seem that the electric current arrests cardiac motion almost instantly, thus preventing further circulation of blood. The necessity for simultaneous analysis of blood and tissue has been pointed out by Graeser, Ginsberg, and Friedemann. It is a valuable aid in the comparison of data from various animals and provides a measure of postmortem changes within the tissues.

Analysis of Blood from Pulmonary Vein.—The left lower lobe was infected in the usual manner. Surgical anesthesia was induced by nembutal. The sternal plate was removed, exposing the heart and lungs. Artificial respiration was immediately begun. Although the number of induced respirations was about 20 per minute the lungs were not fully inflated. While it may have resulted in less complete oxygenation of the blood, this procedure was nevertheless followed for reasons which will be given in the discussion. Blood was withdrawn from the right ventricle, from the pulmonary vein of an uninfected lobe, and from the pulmonary vein of the infected left lower lobe. The blood vessels of this lobe are more accessible and less manipulation is required to obtain the sample. Cold syringes were used, previously oiled and freed of air and fitted with long No. 18 gauge needles. Needles, bent at right angle about 1.5 inches from the tip, were used to obtain the pulmonary blood. A temporary ligature, a loop around the pulmonary vein, may be applied during collection of the sample. The blood was immediately emptied into cold tubes containing oil and the necessary quantities of sodium oxalate and sodium fluoride.

RESULTS

Analysis of Tissues.—Normal lung tissue, perhaps more than any other tissue, carries on its functions in an almost completely aerobic environment. This is especially true of the cells which line the alveoli. The aerobic character of its metabolism is indicated by the following data recalculated from Table I and shown in Table II. Excluding the data from dog 1, the averages from 10 animals expressed as mg. per cent, for the normal lung tissue were glucose 72,[1] lactic acid 15; the averages for blood were glucose 84, lactic acid 15. Assuming that one-fifth of the normal tissue analyzed was blood,[2] the calculated concentrations of glucose and lactic acid within the tissue itself were 69 and 15 mg. per cent respectively. In spite of the great vascularity of the tissue and the rapid diffusion of glucose from the blood into the tissue, the normal tissue contained an average of 14 mg. per cent less of glucose. This indicates a rapid metabolism of glucose. Lactic acid either was not produced at all, or else its rate of production was balanced by its rate of oxidation. Altogether, the results indicate a completely aerobic metabolism of carbohydrate.

Entirely different results were obtained from the consolidated tissue. This was to be expected for the growth of the bacterium brings about a profound change in the environment. Although the number of pneumococci at first may be relatively small, there is an immediate response resulting in engorgement of the capillaries and filling of the alveoli with serous exudate (9, 10). Whether the initial edema represents an anaphylactic reaction or whether it is due to an edema-producing irritant (11) cannot be determined with the data at hand. The growth of the pneumococcus appears to be very rapid at this stage, for many organisms can be seen in the exudate. Almost simultaneously great numbers of polymorphonuclear leucocytes enter the alveoli until the latter become packed with leucocytes; the organisms are at the same time rapidly removed by phagocytosis. With

[1] That the reducing power of zinc filtrates from tissues is due largely to glucose was shown recently by Blatherwick (8). The average values for the non-fermentable reducing substances, expressed as mg. per cent of glucose, were as follows: liver, 11.7; muscle, 12.0; kidney, 7.6; blood, 4.9.

[2] Although the correctness of this assumption, in the absence of hemoglobin determinations, may be questioned, the results are not greatly affected by calculations of this kind, nor are the conclusions materially altered.

TABLE I

Experimental Pneumococcus Lobar Pneumonia in Dogs

Dog	Time after onset of infection	Tissue	Analysis of tissues: Glucose	Analysis of tissues: Lactic acid	Analysis of tissues: Glycogen	Histological appearance of infected tissue*	Fresh exudate cells: Polymorphonuclear	Fresh exudate cells: Mononuclear†	Fresh exudate cells: Living cells
	days		*mg. per cent*	*mg. per cent*	*mg. per cent*		*per cent*	*per cent*	*per cent*
1	2	Infected	28	41		Uniform exudate of pmn. cells			
		Normal	48	41					
		Blood	49	21					
2	2	Infected	41	50		Uneven exudate, predominantly pmn. cells	85	15	74
		Normal	81	21					
		Blood	100	19					
3	3	Infected	46	41	547	Uniform exudate of pmn. cells	86	14	92
		Normal	67	11	136				
		Blood	69	10					
4	3	Infected	80	24		Uniform exudate, predominantly pmn. cells			75
		Normal	81	11					
		Blood	97	11					
5	4	Infected	62	43	227	Uneven exudate, predominantly pmn. cells	90	10	77
		Normal	72	26	186				
		Blood	83	27					
6	4	Infected	63	25	374	Uniform exudate of pmn. cells	79	21	96
		Normal	74	11	145				
		Blood	88	10					
7	4	Infected	52	19		Advanced resolution. Marked macrophage reaction	33	67	94
		Normal	63	14					
		Blood	81	11					
8	5	Infected	58	18		Advanced resolution. Exudate of mononuclear cells	33	67	52
		Normal	58	8					
		Blood	76	11					

* In this column, pmn. refers to polymorphonuclear cells.

† This includes lymphocytes, monocytes, and macrophages.

TABLE I—*Concluded*

Dog	Time after onset of infection	Tissue	Analysis of tissues			Histological appearance of infected tissue*	Fresh exudate cells		
			Glucose	Lactic acid	Glycogen		Polymorphonuclear	Mononuclear†	Living cells
	days		*mg. per cent*	*mg. per cent*	*mg. per cent*		*per cent*	*per cent*	*per cent*
9	6	Infected	60	14		Advanced resolution. Scanty exudate of mononuclear cells	17	83	71
		Normal	72	11					
		Blood	87	12					
10	6	Infected	62	21		" "	13	87	89
		Normal	72	20					
		Blood	72	17					
11	7	Infected	59	27		Advanced resolution. Exudate principally of macrophages	15	84	76
		Normal	80	16					
		Blood	84	17					
12	7	Infected			181	Beginning resolution. Exudate contains pmn. cells and macrophages			
		Normal			123				
		Blood							
13	13	Infected	90	12	133	Normal			
		Normal	97	10	128				
		Blood	102	10					
14		Normal			119	Normal			

the filling of the alveoli by exudate, the metabolism is aerobic only in so far as oxygen can be obtained from the blood supply. The situation is further complicated by the fact that pulmonary blood for the most part represents venous blood, and therefore contains less oxygen. If now the circulation should be diminished (12), as is usually considered to be the case in edematous tissues, the available carbohydrate (free sugar and perhaps also glycogen) is rapidly used up by the leucocytes, pneumococci, and, to a lesser extent, the tissue cells. Toxic products and acids thus accumulate. Should the stasis be too great, or should occlusion of blood vessels occur, the carbohydrate would soon be exhausted and the result would be death and necrosis

of the tissue. The pneumococcus can continue to grow at an apparently undiminished rate even after all of the free sugar is metabolized (Friedemann and Sutliff, 13); its source of energy probably is the protein-sugar, a polysaccharide (14), of which from 250 to 1000 mg. per cent are present in serum.

The extent of the metabolism, and its departure from the normal, in the initial stage of consolidation is strikingly shown by the data from dogs 2 to 6. See Table I. The reducing sugar (glucose) was in every case lower in the infected than in the normal tissue; the lactic acid

TABLE II

Recalculated Data from Table I

Stage of disease	Animals	Tissue	Averages		Recalculated data*			
			Glucose	Lactic acid	Glucose	Lactic acid	Difference over blood	
							Glucose	Lactic acid
			(1)	(2)	(3)	(4)	(5)	(6)
			mg. per cent	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*
Before resolution	2 to 6	Blood	87	16	87	16		
		Normal lung	75	16	72	16	15	0
		Infected lung	58	37	55	39	32	23
During resolution	7 to 11	Blood	80	14	80	14		
		Normal lung	69	14	66	14	14	0
		Infected lung	58	20	55	21	25	7

* These are based upon the assumption that the normal tissue contains 20 per cent and the infected tissue 10 per cent of blood.

content was greatly increased. The averages of all determinations for glucose and lactic acid are shown in Table II. Assuming that one-tenth[2] of the consolidated tissue was blood, the average content of glucose and lactic acid, exclusive of blood was 55 and 39 mg. per cent respectively. Compared with blood, the infected tissue contained an average of 32 mg. less of glucose and 23 mg. more of lactic acid than the blood. About 70 per cent of the glucose was thus converted anaerobically into lactic acid. On the other hand, the normal tissue in the same groups of dogs contained 15 mg. per cent less of glucose and no increase whatever of lactic acid. The increased sugar con-

sumption was no doubt due to the great number of leucocytes and pneumococci in the exudate. The polymorphonuclear leucocytes metabolize glucose at an extremely rapid rate and, according to Barron and Harrop (15), produce large quantities of lactic acid even in a completely aerobic environment. Yet in spite of the greatly increased metabolism the tissues still contained carbohydrate and a relatively slight increase in the concentration of lactic acid. It is apparent that the circulation of blood is sufficient to allow ample carbohydrate to diffuse into the tissue and, by diffusion outward, to remove the metabolic products.

The recovery was accompanied by a marked macrophage reaction, in confirmation of the studies by Robertson (9, 10, 16) and Kredel and Van Sant (3). From Table I it can be seen that the mononuclear cells (mostly macrophages), which at first constituted only about 10 per cent of the exudate cells, increased in numbers until at the time of resolution they constituted more than two-thirds of all cells. At the time of analysis the tissues still contained some exudate and many cells, as was shown by x-ray and by microscopic examination of sections. The condition of the animals was greatly improved. The individual data in Table I still show a higher metabolism of carbohydrate in the infected than in the normal tissue. This was to be expected since the exudate still contained many cells. On the other hand, the lactic acid content was reduced. This is best shown by the recalculated data from dogs 7 to 11 in Table II. The average difference between the reducing sugar of blood and tissue is 25 mg. per cent; the difference in lactic acid content is only 7 mg. per cent. This, we believe, points to an almost complete reestablishment of aerobic conditions. A certain amount of lactic acid is still to be expected, since the diffusion of oxygen from the blood into the exudate contained in the alveoli is not rapid enough to prevent entirely lactic acid formation. But even if the environment were completely aerobic a certain amount of lactic acid would still be formed, for Barron and Harrop (15) have shown that macrophages produce some lactic acid from sugar in an aerobic environment.

Analysis of Blood from Pulmonary Vein.—Results from two animals are shown in Chart 1 and Tables III and IV.

It was realized in these experiments that the conditions could not be the same as in the intact animal. They were decidedly abnormal due to the deep nembutal anesthesia, a rapid loss of blood (some of which was unavoidable because of the operative procedure), and to the under-ventilation. The lungs were under-ventilated (*a*) to avoid unnecessary manipulation of the heart and lungs during

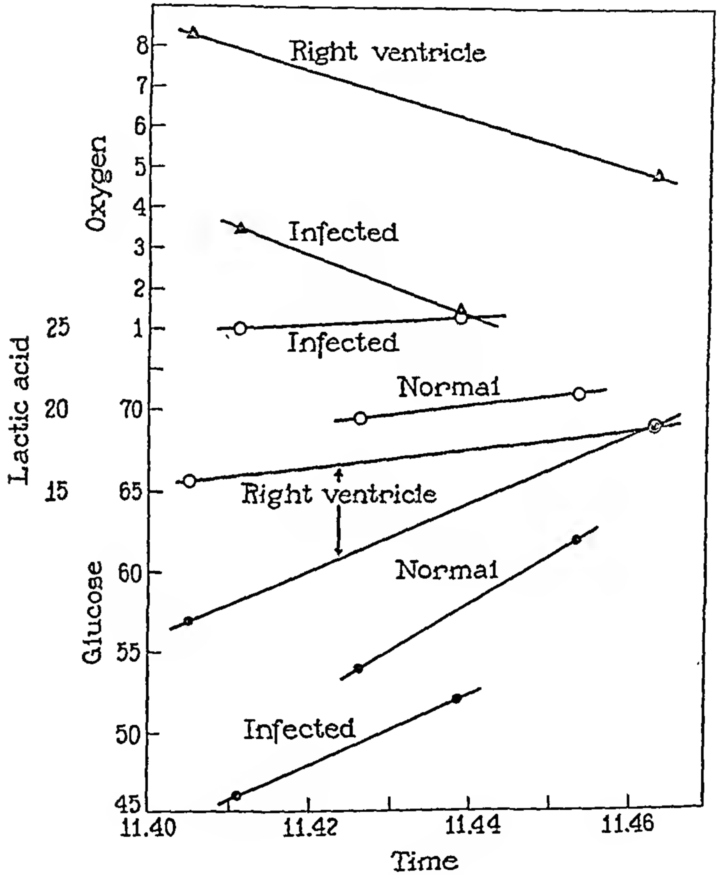

CHART 1. Dog 15. Analysis of blood from the right ventricle and from the normal and infected lobes.

sampling of blood and (*b*) to produce if possible a high degree of oxygen unsaturation of the blood. The latter provided a method of determining whether the metabolism of pneumonic lung tissue could proceed in a normal manner even at low oxygen tensions. Considering the results from both animals, it will be noted first of all that the sugar content of the blood increased in the course of the ex-

periment. This was accompanied by parallel increases of lactic acid.[3] Because of these constant increases it was necessary to collect more than one sample from each site and to note the exact time of collection of each sample.

Dog 15 (see Chart 1) was examined 48 hours after infection. The left lower lobe was completely consolidated and the upper lobes on the same side showed considerable involvement. The entire right lung was normal as was determined by immediate inspection and later by microscopic section. The blood entering the lungs contained only from 8.3 cc. oxygen per 100 cc. at the beginning of the experiment to 4.9 cc. at the end.[4]

TABLE III

Calculations Based upon Interpolated Data from Chart 1 at 11:43

	Right ventricle blood entering lungs	Blood from normal lobe	Blood from consolidated lobe
Glucose, *mg. per 100 cc.*	62	55	50
Lactic acid, *mg. per 100 cc.*	17.0	19.5	25.5
Oxygen, *cc. per 100 cc.*	6.8		2.1
Glucose metabolized, total, *mM per liter*		0.39	0.67
Glucose changed to lactic acid, *mM per liter*		0.08	0.47
Glucose oxidized, *mM per liter*		0.31	0.20
Glucose oxidized, *per cent of total*		80	30
Oxygen consumed, *mM per liter*			2.1
" required by 0.20 mM glucose, *mM per liter*			1.2
" consumed by other metabolites (fats, etc.), *mM per liter*			0.9

Calculations, based upon interpolated data from Chart 1 at 11:43, are shown in Table III. At this time blood from the pulmonary vein of the normal lobe contained 7 mg. per cent less of glucose than the blood entering the lung. The lactic

[3] The hemoglobin content also increased. Thus, in the case of dog 15, the oxygen capacity at 11:40 was 21.8 cc. per 100 cc. and had increased to 31.5 cc. at 11:46. A similar rise was also observed in dog 16, where the oxygen capacity rose from 21.5 cc. at 11:51 to 24.9 cc. at 11:57. This indicates a rapid loss of plasma into the tissues, due perhaps to shock.

[4] It should be remembered that this represents venous blood from the body tissues. The oxygenation in the normal lung was apparently excellent. The oxygen content at the end of the experiment was 29.5 cc. per 100 cc. as compared with an oxygen capacity of 31.5 cc. of blood taken 1 minute later. However the combined blood from the lungs, *i. e.*, mixed blood taken from the left ventricle, at the end of the experiment, contained only 17.7 cc.

acid content was increased only slightly above the experimental error of the method. However, assuming the determined lactic acid value as correct, 80 per cent of the sugar was aerobically metabolized; only 20 per cent appeared anaerobically as lactic acid. This confirms the conclusions reached from the analysis of normal tissue.

The blood from the consolidated lobe still contained a large part of the glucose, while the lactic acid was only moderately increased. The sugar consumption was greater than in the normal tissue, as indicated by a decrease of 12 mg. per cent, and a lactic acid increase of 8.5 mg. per cent. Such an increase was to be expected, because of the large numbers of leucocytes. The metabolism was largely anaero-

TABLE IV

Analysis of Blood 5 Days after Infection, Dog 16

Recovering. Infected lobe ⅓ to ½ consolidated. Blood taken from pulmonary veins.

Blood	Average time of collection	Glucose	Lactic acid	Oxygen	
				Content	Capacity
	hrs.:min.:sec.	*mg. per cent*	*mg. per cent*	*cc. per cent*	*cc. per cent*
Right ventricle	11:51:11	79.5	16.9	14.6	21.5
Infected lobe	11:53:36	72.5	19.6	21.1	
Normal "	11:56:02	72.5	22.9		
Right ventricle	11:56:50	85.0	25.0	14.7	24.9
Infected lobe	11:58:34	76.0	28.9	15.4	
Normal "	11:59:45	85.2	33.2		
Right ventricle	12:00:35	99.5	35.0	11.4	
Infected lobe*	12:06:30	92.7	39.2		
Right ventricle	12:09:47	104.7	44.1		

* Ligature was applied around the pulmonary vein while blood was drawn. It was then immediately released.

bic, due no doubt to the leucocytes. In almost exact agreement with the results from tissues, only 30 per cent of the glucose was oxidized; 70 per cent appeared as lactic acid. Calculated in terms of millimols per liter (see Table III), 0.67 mM of glucose were metabolized, of which 0.47 mM were converted into lactic acid. Glucose oxidized was therefore 0.20 mM. The latter required 0.20 x 6, or 1.2 mM of oxygen for complete oxidation. Our analyses, however, indicate an oxygen consumption of 2.1 mM. This leaves a balance of 0.9 mM for the oxidation of other metabolites.

Dog 16 was studied 5 days after infection. This animal was recovering, as indicated by the x-ray examination, the drop in temperature, and the general improvement. The infected lobe was still one-third to one-half consolidated, but

these areas contained considerable air, as could be judged by the extent of inflation and the later microscopic section. The results fluctuated somewhat, especially those from the infected lobe. Thus at 11:53 the oxygen content of the blood from the infected lobe was 21.1 cc. per 100 cc., indicating almost complete oxygenation, while at 11:58 the oxygen content was slightly higher than the blood entering the lung. Since passage through atelectatic lung tissue, as shown by Adams (17), and through consolidated tissue as shown above by us, results in loss of oxygen, it is apparent that these results indicate access of air. The high degree of oxygenation at 11:53 and the low oxygenation at 11:58 is no doubt due to the method of sampling which in the one took blood from a branch of the pulmonary vein coming from a normal area, while the other represented blood from a not quite recovered area. When plotted, the results indicate a somewhat higher sugar consumption by the normal lung tissue of this dog as compared with dog 15, an average of 11 mg. The blood from the infected lobe contained an average of 13 mg. less of glucose. All of the lactic acid data, within the limits of the method, fall on the same curve. Thus, as was also concluded from the tissue analyses, the recovering tissue consumes only slightly more glucose than the normal tissue, and the results as a whole indicate a return to a completely aerobic metabolism.

DISCUSSION

An important factor in the recovery is the marked change in the type of cells which precedes resolution. This phenomenon has long been known (18). Its relation to the process of resolution and its probable function have been investigated recently by Robertson and coworkers (10, 16). It will be noted from Table I that the relative number of mononuclear cells[5] increased after the 3rd day. Menkin (19), who studied this change in exudates, has suggested that it is due to a rapid depletion of sugar and the development of a high local acidity, all of which may damage the leucocytes; the latter are then replaced by macrophages which are capable of metabolizing under the less favorable conditions. This suggestion is based upon the changes in sugar and lactic acid content and the pH of pleural fluid following injections of turpentine. The exudate, following the initial small dose of turpentine as administered intrapleurally by Menkin, contains mainly leucocytes; no marked accumulation of lactic acid or lowering of the pH is observed. On the 2nd or 3rd day the same or a larger dose of turpentine is administered. This is followed almost immediately by a marked drop in the sugar content, an increase in the lactic

[5] This includes lymphocytes, monocytes, and macrophages.

acid, and a decrease of the pH below 7.0. Macrophages are found to predominate on the succeeding day. The time interval at which the reaction occurs should be noted.[6] Also to be noted in experiments of this kind is the fact that the volume of pleural fluid is large as compared with the absorbing surface; therefore, a rapid interchange between the exudate and the blood is not to be expected. In our experiments, on the other hand, the volume of alveolar exudate is small as compared with the absorbing surface, and the circulation, as we have shown, is adequate to maintain normal metabolism. The number of viable cells, as shown in the last column of Table I, is about the same throughout. Leucocytes are not apparently injured by the conditions which obtain during the first 2 or 3 days, and they disappear at the time when the conditions are returning to normal. Perhaps of greater significance than any possible hydrogen ion changes, is the more prompt macrophage response found in the lungs of animals which have recovered from previous pneumococcus infections (10).

Various factors no doubt contribute to the uneventful and perfect recovery of lung tissue. The pneumonic infection differs in some respects from infections in other tissues; the exudate accumulates in the alveoli, while the tissue itself is relatively little involved. Yet at times many organisms may be seen within the parenchyma, and the latter appears to be decidedly thickened. Toxic substances (20-23, 11) are probably formed by the pneumococcus, but their effect does not seem to be as severe as that of the toxic products of other bacteria. Our results show that the blood supply throughout provides oxygen (even though the latter must be obtained from venous blood) and nutrients, and prevents the accumulation of waste products. The metabolism is thus practically normal throughout, even though the conditions under which the tissue metabolizes have been radically changed. The maintenance of a fairly normal condition throughout is perhaps the most important factor in the complete restoration of the infected tissue.

SUMMARY AND CONCLUSIONS

The metabolism of infected and uninfected lung tissues was determined at various stages of experimental lobar pneumococcus

[6] It is well known that macrophages do not appear in large numbers in exudates induced by such substances as gum arabic until the 2nd or 3rd day.

pneumonia in dogs. Analyses of tissues and analyses of the blood entering and leaving the lungs indicate a fairly normal aerobic metabolism of the tissue throughout the course of the infection.

We wish to express our appreciation of the help and suggestions given by Dr. O. H. Robertson in the course of this work.

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CORRECTION

In Vol. 67, No. 2, February 1, 1938, page 237, Table IV, in the heading of the first column, for $\underline{10^{-6}}$ read $\underline{10^{6}}$.

SOME EFFECTS OF FORMALDEHYDE ON HORSE ANTIPNEUMOCOCCUS SERUM AND DIPHTHERIA ANTITOXIN, AND THEIR SIGNIFICANCE FOR THE THEORY OF ANTIGEN-ANTIBODY AGGREGATION

By HARRY EAGLE, M.D.

(From the Syphilis Division, Department of Medicine, Johns Hopkins Medical School, Baltimore, and the United States Public Health Service, Washington, D. C.)

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It has been shown in a previous communication (1) that diazo compounds added to antipneumococcus horse serum or to horse diphtheria antitoxin cause a dissociation between the aggregating activity of the antibody *in vitro* and its protective action *in vivo*. When a small amount of sodium diazosulfanilate was added to diphtheria antitoxin, the latter no longer gave the Ramon flocculation reaction with toxin, but its ability to combine with toxin, and its protective action *in vivo* were unaffected. Similarly, when antipneumococcus serum was coupled with a small amount of diazo compound, the azoprotein dye so formed no longer gave the characteristic precipitation with the type specific capsular carbohydrate, but its bacterial agglutinating activity was only slightly affected, and its protective action *in vivo* not at all. A larger amount of diazo compound added to the antipneumococcus serum caused an apparent loss of its agglutinating activity; but if the mixture of treated serum and bacteria was centrifuged, the pressure packing of the sedimented bacteria caused their cohesion to form the characteristic flake of agglutinated pneumococci. At this stage, the serum still protected mice. On further treatment with diazo compound all antibody activity progressively decreased, and eventually disappeared.

It was subsequently shown (2) that the coupling of protein with diazo compounds was a complex reaction in which not only the dye-forming histidine NH and tyrosine OH groups might conceivably be

involved, but also the aliphatic NH_2 groups, and the NH groups of proline and arginine. It remained to ascertain which of these five groups was primarily concerned in the dissociation of antibody activity just described, the reason for this dissociation and its significance for the mechanism of antigen-antibody aggregation.

The simplest experimental attack seemed to be the study of the effect of formaldehyde on the activity of antipneumococcus serum and diphtheria antitoxin. Formaldehyde apparently does not react with the proline or the arginine NH group in protein (3). Of the five groups in protein previously found to react with diazo compounds, it is said that only two, the aliphatic NH_2 (4) and the histidine NH (5), are readily affected by formaldehyde. The present experiments were therefore undertaken to ascertain whether the effects of diazo compound previously described could be duplicated with formaldehyde, and thus, could be reasonably ascribed to modifications in either the aliphatic NH_2 or histidine NH of the antibody molecule.

It is a well known observation that formaldehyde in concentrated solution destroys antibodies (6). Chow and Geobel (7) have recently shown that under certain conditions the inactivation of antipneumococcus globulin by formaldehyde is reversible, presumably due to the hydrolysis of $—N{=}CH_2$ groups formed on the addition of formaldehyde. Several investigators (8) have reported the variable susceptibility of different antisera to the destructive action of formaldehyde. Mudd and Joffe (9), in a study which is particularly germane to the experiments here to be reported, found that agglutinating sera treated with an equal volume of 9 to 37 per cent HCOH lost some of their activity, and showed wide prozones in the agglutination reaction. In the presence of an excess of antiserum there was no obvious agglutination, but cohesion was obtained on centrifugation. That combination with antibody has occurred was further shown by the change in the cataphoretic properties of the organisms. A similar decrease in the agglutinating tendency was observed if the bacteria were first sensitized in untreated antiserum, and if the washed bacteria were then treated with formaldehyde.

As will be shown in the present paper, the bizarre effects of diazo compounds on diphtheria antitoxin and antipneumococcus serum could be reproduced with formaldehyde. A minute amount sufficed

to inhibit the aggregating activity of these sera completely. Although the reaction between HCOH and protein is complex, it seems possible that this inhibiting action on aggregation is primarily due to the modification of a few NH_2 groups in the antibody molecule. The reason for this inhibition, and the implications of these observations with respect to the mechanism of antigen-antibody aggregation are discussed in the text. In contrast to the effect on aggregation, even large quantities of formaldehyde did not affect either the ability of these two antibodies to combine with antigen *in vitro*, or their protective action *in vivo*. It follows that the aliphatic NH_2 groups of diphtheria antitoxin and antipneumococcus serum are not primarily concerned in their combination with the homologous antigens.[1]

EXPERIMENTAL[2]

The Effect of Formaldehyde on Diphtheria Antitoxin

Varying amounts of formaldehyde[3] were added to fixed amounts of diphtheria antitoxin, as indicated in Table I. After 1 hour at room temperature the mixtures were dialyzed in cellophane tubing against running water for 24 hours,[4] made isotonic by the addition of 1/19 volume of 17 per cent NaCl, adjusted to pH 7.0, and tested for antibody activity.

As shown in Table I, 1 part of formaldehyde solution to 2048 parts of serum, acting for 1 hour at room temperature, definitely retarded the Ramon flocculation reaction with toxin, and 1 part to 64 parts of serum prevented flocculation completely. In marked contrast, a 1:8

[1] It should be emphasized that although the formolized antibody might conceivably be reversed to native antibody *in vivo*, such dissociation does not occur under the conditions of the *in vitro* experiment. The formolized antibody itself combines with its antigen in the test tube (*cf.* page 499).

[2] I am indebted to the Mulford Biological Laboratories, Glenolden; the Eli Lilly Company, Indianapolis; the Lederle Laboratories, Pearl River; and the Health Departments of Massachusetts, New York City and New York State for the antisera, refined globulin and diphtheria toxin used in these and subsequent experiments. The Mulford Biological Laboratories also furnished preparations of acetylated Type I and Type II pneumococcus carbohydrate.

[3] Merck reagent, containing approximately 37 per cent HCOH.

[4] In some of the early experiments, the formaldehyde was almost instantaneously inactivated after the desired interval by the addition of an excess of $NaHSO_3$. The results did not differ from those obtained on dialysis.

TABLE 1

The Effect of Formaldehyde Acting for 1 Hour on the Flocculating Activity and Protective Action of Horse Diphtheric Antitoxin

Antitoxin serum	37 per cent HCOH	Ratio of HCOH: protein		M/1 NaOH necessary to neutralize*	Approximate number of formolized NH_2 groups in antibody molecule per 100,000 molecular weight†	Final volume after dialysis and addition of NaCl	A Ramon flocculation (figures indicate flocculation time) Varying amounts of treated 1:2 serum + 1 cc. toxin						B Protective action in guinea pigs Varying amounts of serum + 1 L+ dose toxin							C Conclusion
		Concentration	Molar				0.2 cc.	0.1 cc.	0.075 cc.	0.05 cc.	0.0375 cc.	0.025 cc.	0.02 cc.	0.012 cc.	0.009 cc.	0.006 cc.	0.0045 cc.	0.003 cc.	0.002 cc.	
cc.	cc.			cc.		cc.	min.	min.	min.	min.	min.	min.								
4	0	0	0	—		8	0	Cl	180	37	20	130	S	S	S	S	S, D2	D2	D1	Progressive retardation of Ramon flocculation, with eventual complete disappearance. The protective action is unaffected even by 128 times the quantity which causes a significant retardation of flocculation
4	0.001	1:4096‡	3.75:1§	—		8	0	Cl	180	42	24	180								
4	0.002	1:2048	7.5:1	—		8	0	Cl	180	75	39	180								
4	0.004	1:1024	15:1	—		8	0	Cl	180	180	63	180								
4	0.008	1:512	30:1	—		8	0		Cl	180	180	180								
4	0.016	1:256	60:1	0.03	10	8	0	0	0	180	180	Cl								
4	0.031	1:128	120:1	0.05	15	8	0	0	0	180	180	0	S	S	S	S	D4	D2	D1	
4	0.062	1:64	240:1	0.07	22	8	0	0	0	0	0	0								
4	0.125	1:32	480:1	0.11	35	8				0			S	S	S	S	D4	D1	D1	
4	0.25	1:16	960:1	0.14	43	8				0			S	S	S	S	D2	D3	D1	
4	0.5	1:8	1920:1	0.14	43	8				0			S	S	S	S	D4	D2	D1	Progressive, but incomplete, destruction of protective action
4	1	1:4	3840:1	0.145	46	8				0			S	S	S	S, D5	D2	D1	D1	
4	2	1:2	7680:1	0.15	47	8				0			S	S	S	D4	D1			
4	4	1:1	15,360:1	0.16	50	8				0			S	S	D4	D1	D1			

0 = no flocculation in 24 hours. Cl = cloudy after 24 hours at 37°C. S = survived more than 4 days; D1 = dead in 1 day, etc.

* Back to original serum pH.

† Approximate number of NH_2 groups $= \frac{\text{cc. 1 M NaOH}}{\text{cc. serum}} \times \frac{\text{molecular weight protein}}{\text{gm. serum protein per liter serum}}$.

$= \frac{\text{cc. 1 M NaOH}}{4} \times \frac{100{,}000}{80} = 312.5 \times \text{cc. 1 M NaOH}.$

‡ 1 part of 37 per cent HCOH to 4096 parts of serum.

§ 3.75 moles of HCOH to 1 mole of serum protein. See footnote 5, page 499.

ratio had no demonstrable effect on the protective action of the antiserum *in vivo*, as tested with guinea pigs; and even a 1:1 ratio did not wholly destroy the antitoxin. The partially treated antiserum was clearly capable of neutralizing toxin *in vivo*, despite the absence of the usual aggregation.

It is to be noted in Table I (section A, bold-faced column headed by 0.0375 cc.) that as the amount of formaldehyde was increased, the toxin: antitoxin ratio which gave the most rapid flocculation did not significantly vary, despite the progressive retardation of that flocculation. Since this optimum ratio is the index of the "neutral" mixture, in which toxin and antitoxin are combined in "equivalent" proportions, it follows that the ability of the antitoxin to combine with toxin was unaffected by the treatment with formaldehyde. This was further shown by the fact that a rabbit antiserum to horse serum protein, added to a non-flocculating and non-toxic mixture of antitoxin and toxin, precipitated both the antitoxin protein and the toxin with which it had combined, and left a non-toxic supernatant fluid (*cf.* 10). A control mixture of formaldehyde-treated antipneumococcus serum and diphtheria toxin, similarly precipitated by a rabbit antiserum to horse serum, yielded a supernatant fluid of undiminished toxicity.

If we assign an arbitrary figure of 100,000 as the "average" molecular weight of serum protein, and assume that the antibody protein does not significantly differ in its affinity for formaldehyde from the rest of the serum protein, it follows that an amount of formaldehyde sufficient to combine with approximately 7 to 8[5] NH_2 groups in each molecule of serum protein, and which probably reacted with no more than 2 or 3 groups in the course of 1 hour (*cf.* sixth column of Table I), significantly retarded the flocculating activity of diphtheria antitoxin with toxin. As determined with a glass electrode, this amount of

[5] If we assume an average molecular weight of 100,000 for all the serum proteins, a serum containing 8 per cent protein is 0.0008 M. A 37 per cent solution of HCOH is approximately 12.3 M. 1 part of that solution to 2048 parts of serum is a $\frac{12.3}{0.0008 \times 2048}$, or approximately an 8:1 ratio. The actual number of groups of the antitoxin molecule which might be affected by a given amount of HCOH differs from this calculated value to the extent that the molecular weight of the antitoxin protein itself differs from the arbitrarily chosen average of 100,000.

HCOH had no demonstrable effect on the pH of the serum, further evidence that but few NH_2 groups had been affected. Under the same conditions, the protective action of the antiserum was wholly unaffected even by 250 times that quantity. As shown by the amount of NaOH required to neutralize (*cf.* fifth column of Table I), the latter amount of HCOH sufficed to block practically all the free NH_2 groups of the protein. As was concluded by Mudd and Joffe (9) for antibodies to various bacteria, it seems clear that the aliphatic NH_2

TABLE II

The Effect of Formaldehyde on the Antigenic Activity of Horse Diphtheric Antitoxin, as Determined by Its Reactivity with a Rabbit Antiserum vs. Horse Serum

(Antitoxin treated with formaldehyde as shown in Table I)

Ratio of 37 per cent HCOH to serum	A Varying amounts of the 1:2 treated serum + 0.4 cc. rabbit antiserum + NaCl up to 0.8 cc. Figures represent degree of precipitation after 4 hrs. at 37°									B Supernatant fluids from section A + 0.01 cc. fresh horse serum. Figures represent precipitation after 2 hrs. at 37° (test for free antibody)	Conclusion
	0.4 cc.	0.2 cc.	0.1 cc.	0.05 cc.	0.025 cc.	0.0125 cc.	0.0002 cc.	0.0031 cc.	0.0016 cc.		
0	3	4	4	4	4	4	4	3	2	0 ± 2 3 4 4 4 4 4	Only slight change in the precipitating activity of partially formolized antitoxin (Table I), acting as antigen with a rabbit antiserum *vs.* horse serum. Marked precipitation prozone
1:32	±	2	4	4	4	4	4	3	2	0 0 2 3 4 4 4 4 4	
1:16	0	1	4	4	4	4	4	3	2	0 0 2 3 4 4 4 4 4	
1:8	0	0	2	4	4	4	4	3	2	0 0 1 2 4 4 4 4 4	
1:4	0	0	1	4	4	4	4	3	2	0 ± 1 3 4 – – – –	
1:2	0	0	1	3	4	4	4	3	2	0 ± 2 3 4 – – – –	
1:1	0	0	0	±	3	3	3	3	2	0 ± 2 3 4 – – – –	

groups play little or no rôle in the combination between diphtheria toxin and horse antitoxin.

It is to be noted (Table II) that the antigenic activity of horse antitoxin, that is, its reactivity as horse serum with a precipitating rabbit antiserum to horse serum, was as little affected by formaldehyde as was its antitoxic activity. An 18 per cent concentration of HCOH acting for 1 hour had little effect on its precipitating activity, save for a wider prozone in the region of antigen excess; and the formaldehyde had even less effect on its combining affinity for the antibody, as

TABLE III

The Effect of Formaldehyde on the Antitoxic and Antigenic Activity of Horse Diphtheric Antitoxin

Time for which equal volumes of 57 per cent HCOH and antitoxin were allowed to react*	pH of reacting mixture (brought to pH 8.85 after 1 hr.)	Activity of treated serum as antibody. Protective action in guinea pigs. Varying amounts of 1:2 treated serum + 1L + dose of toxin											A. Precipitating activity of treated serum as antigen with rabbit antiserum *vs.* horse serum. Varying amounts of treated 1:2 serum + 0.4 cc. antiserum in total volume of 0.8 cc.								B. Supernatant fluids from section A + 0.01 cc. untreated horse serum (test for free antibody)	C. Anaphylaxis experiments with guinea pigs sensitized to native horse serum. Amount of treated 1:2 serum injected		Conclusion
		0.2 cc.	0.15 cc.	0.1 cc.	0.175 cc.	0.05 cc.	0.0375 cc.	0.025 cc.	0.018 cc.	0.125 cc.	0.009 cc.	0.006 cc.	0.4 cc.	0.2 cc.	0.1 cc.	0.05 cc.	0.025 cc.	0.125 cc.	0.0062 cc.	0.0031 cc.		2 cc.	1 cc.	
hrs.																								
0†								S	S	S	S	D4	4	4	4	4	4	4	3	3	0 0 ± 2 3 4 4 4	D, D	D, D	Progressive slow de[…] in the protective […] of formolized […] toxin, its precipi[…] activity with a […] antiserum, an[…] ability to shock […] pigs sensitized […] native horse ser[…]
½	8.83					S		S	S	D1			0	0	1	2	2	2	2	1	0 1 4 4 4 4 4 4	D, scratch dyspnea, convulsions		
1½	8.81			S				S	S	D2			0	0	1	2	2	2	1	±	± 1 4 4 4 4 4 4			
3	8.80			S		S	S	D1					0	0	0	±	1	1	±	0	± 2 4 4 4 4 4 4			
6	8.80	S				S, D4	D3	D2					0	0	0	0	±	±	±	0	1 3 4 4 4 4 4 4	Scratch, dyspnea, prostration (2 animals)	Scratch, dyspnea (2 animals) 0 (slight weakness) (3 animals)	
12	8.80	S	S	D3		—	D1						0	0	0	0	0	0	0	0	3 4 4 4 4 4 4 4			

S = survived more than 4 days; D1 = dead in 1 day, etc.

* It is obvious that all these times are in error to the extent that the formaldehyde was not removed instantaneously on dialysis. Howeve[…] concentration probably fell to less than ½ its original level within an hour.

† Original serum, diluted with equal volume NaCl.

shown by the subsequent addition of untreated horse serum (Table II, section B). Like the antitoxic activity, the species specificity of horse serum protein apparently does not depend primarily on its free NH_2 groups.

It is true that this concentration of formaldehyde, acting over a 24 hour period, eventually almost completely destroyed the protective action of the antitoxin serum, as well as its reactivity with an antibody to horse serum (Table III). However, this destruction cannot be ascribed to the simple addition of HCOH to the NH_2 groups of protein. The latter reaction proceeded very rapidly in the presence of so large an excess of HCOH, as evidenced by the approximately constant pH of the reacting mixture after the first hour. The destruction of antibody, on the other hand, was incomplete even after 12 hours. Some reaction other than the blockade of the NH_2 groups is apparently responsible for this slow destruction.

The loss of flocculating activity caused by small amounts of formaldehyde is probably due to its addition to a few aliphatic amino groups.[6] The minute amounts which suffice (too small even to affect the pH of the serum), and the speed with which the inactivation may proceed,[7] both suggest that this is the case. Nevertheless, in view of the complexity of the reaction between HCOH and protein the possibility of some other reaction must be considered.

It seems possible that the loss of Ramon flocculating activity frequently observed in the course of concentrating and refining diphtheric antitoxin globulin, may be due to a similar modification of relatively few groups, perhaps the NH_2 groups. Thus, as is seen in Table IV, when antitoxin serum of pH 9.4 to 10.0 was kept at 56°C. for 1 to 4 hours there was a significant retardation or even loss of Ramon flocculation, without any change either in the optimum

[6] The total number of amino acid NH_2 groups incorporated in globulin far outnumbers those of histidine NH, which constitutes only 2.8 per cent of the serum protein (11 *a*). If we assume that the number of free NH_2 groups in protein bears a similar relationship to the number of histidine NH groups capable of reacting with formaldehyde, and if we assume an equal reactivity with formaldehyde, it follows that the first few groups in the antibody to react with HCOH are the NH_2 rather than the NH.

[7] Almost instantaneous with *e.g.*, a 1:20 ratio of formaldehyde:*serum*.

toxin:antitoxin ratio, or in the protective action of the preparation *in vivo*. Similar heating at pH 5.8 to 6.8 had only a slight effect on the flocculation reaction. It is of interest that a similar loss of precipitation and agglutination, with no impairment of protective action. was noted by Felton and Bailey (11 *b*) on heating antipneumococcic serum for ½ hour at 56°C.

TABLE IV

The Effect of Heating at 56°C. on Ramon Flocculation Time of Diphtheric Antitoxin Serum

Antiserum No.	pH before heating	Heating at 56°C. — 0	1 hr.	2 hrs.	4 hrs.	pH after heating at 4 hrs. at 56°C.
		Optimum flocculation time with toxin				
		min.	*min.*	*min.*	*min.*	
1	9.36	75	95	100	210	—
	5.8 to 6.1	67		80	80	
2	9.57	70	180	360	600	9.3
	5.8 to 6.1	65		80	95	
3	10.1	95	180	95	105	9.35
	5.8 to 6.1	65	—			
4	9.58	65	180	420	1440	—
	5.8 to 6.1	65	—	80	—	
5	9.4	60	140	420	—	9.0
	5.8 to 6.1	55	—	100	—	
6	10.05	75	420	1440		—
	5.8 to 6.1	65	—	100	—	
7	9.78	70	420	420	1440	9.4
	5.8 to 6.1	65		125	120	

The Effect of Formoldehyde on Antipneumococcus Serum

In the case of a mixed Type I and II antipneumococcus serum, 1 part of 37 per cent formaldehyde to 2048 parts of serum, acting for 24 hours at room temperature, largely inhibited its precipitating activity with the type specific capsular carbohydrates; and a 1:1024 ratio

TABLE [illegible]

The Effect of Formaldehyde Acting for 24 Hours on the Type I Precipitating, Agglutinati[illegible]

Antiserum	37 per cent* HCOH	Molar ratio of HCOH: protein†	M/1 NaOH necessary to neutralize	Approximate number of formolized NH_2 groups in antibody molecule per 100,000 molecular weight‡	A Precipitation of type specific acetyl polysaccharide: Varying amounts of treated serum + 0.2 cc. 1:400,000 acetylated SSSI								Supernatant of previous section + 0.2 cc. untreated serum (test for free carbohydrate)								B Agglutination [illegible]: Varying amounts of treated serum + 0.2 cc. bacterial suspension: 4 hrs. at 37°C.					
					0.8 cc.	0.4 cc.	0.2 cc.	0.1 cc.	0.05 cc.	0.025 cc.	0.0125 cc.	0.0062 cc.									0.8 cc.	0.4 cc.	0.2 cc.	0.1 cc.	0.05 cc.	0.025 cc.
cc.	cc.		cc.																							
12.8	0	0	0	—	4	4	4	3	2	1	±	0	0	0	0	4	4	4	4	4	4	4	4	4	0	0
12.8	0.0062	7.5:1	0.03	3	Cl	Cl	Cl	0	0	0	0	0	Cl	Cl	Cl	4	4	4	4	4	3	4–	4–	±	0	0
12.8	0.0125	15:1	0.09	8	0	0	0	0	0	0	0	0	Cl	Cl	Cl	2	4	4	4	4	1	1	1	0	0	0
12.8	0.025	30:1	0.14	14									0	0	0	Cl	2	4	4	4	±	±	±	0	0	0
12.8	0.05	60:1	0.21	21									0	0	0	0	Cl	2	2	4	0	0	0	0	0	0
12.8	0.1	120:1	0.28	27	No precipitation in any								0	0	0	2	4	4	4	4	0	0	0	0	0	[illegible]
12.8	0.2	240:1	0.37	36									0	0	2	4	4	4	4	4						
12.8	0.4	480:1	0.42	41									±	2	4	4	4	4	4	4	No agglutinati[illegible]					
12.8	0.8	960:1	0.44	43																						
12.8	1.6	1920:1	0.46	45									1	2	4	4	4	4	4	4						
12.8	3.2	3840:1	0.49	48									2	3	4	4	4	4	4	4						
12.8	6.4	7680:1	0.5	49									2	4	4	4	4	4	4	4						
12.8	12.8	15,360:1	0.51	50									2	4	4	4	4	4	4	4						

Cl = cloudy; numbers 1 to 4 represent increasing degrees of precipitation after 4 hours at 37[illegible]

S = survived; 1 = dead in 1 day, etc.

* Allowed to act overnight at room temperature.

† Assuming a molecular weight of 100,000 for serum protein, serum containing 8 per cent p[illegible]

‡ See †, Table I and footnote 5, page 499.

V

ing and Protective Action of a Mixed Type I and Type II Antipneumococcus Serum

bacteria	C Protection of mice								Conclusions
Readings after mild centrifugation	Varying amounts of treated 1:2 serum + 0.1 cc. pneumococcus culture								
	1.6 cc.	0.8 cc.	0.4 cc.	0.2 cc.	0.1 cc.	0.05 cc.	0.025 cc.	0	
4 4 4 4 4 0 0	2SSSS		1SSSS	SSSSS	4SSSS	223SS	22345	11111	Carbohydrate-precipitating activity of antiserum inhibited; combining affinity *unaffected*
4 4 4 4 4 0 0									
4 4 4 4 4 0 0									Agglutination inhibited; but antiserum can still combine with organisms, as shown by centrifuge agglutination, and can still combine with carbohydrate. Marked decrease in protective action
4 4 4 4 4 0 0									
4-4-4-4-4-0 0	134SS	11123	11233		22333				
4-4-4-4-0 0 0	1112	11111		12222					Progressive decrease, and eventual disappearance of *combining affinity for carbohydrate*, centrifuge agglutination and protective action. Activity of antiserum acting as antigen in guinea pigs sensitized to horse serum also impaired (*cf.* Table VI)
2 ± 0 0 0									
0 0 0 0 0									
No agglutination									

ollowed by 18 hours at 2°C.

ı is 0.0008 M; commercial formaldehyde is approximately 12 M.

prevented precipitation entirely. However, as is shown in Tables V and VI, the treated antibody could still combine with the carbohydrates.[8] On the addition of normal antibody to a non-precipitating mixture, no precipitation was observed; the carbohydrate had apparently been found by the treated antibody, but the secondary aggregation had been somehow prevented. The rough measure of combining affinity illustrated in section A of Tables V and VI revealed no demonstrable decrease.

At this stage the treated serum could still agglutinate bacteria. Larger amounts of 37 per cent formaldehyde (1 part to 64–256 parts serum) caused an apparent loss of agglutinating activity. On centrifugation, however, the bacteria cohered to form the characteristic flake. The treated antibody could apparently still combine with the bacteria, and its activity in this respect was not significantly less than that of the original serum, as shown by centrifuge agglutination. However, the surface deposit of antibody protein was apparently less conducive to aggregation than normally, and it required the pressure packing of the centrifuge to produce cohesion.

With larger amounts of formaldehyde, there was a progressive decrease and eventual disappearance of both centrifuge agglutination and protective action. As long as the antibody could cause spontaneous agglutination, it was capable of protecting mice; but when the protein had been so altered that centrifugation was required in order to produce aggregation, its protective action was definitely impaired.

These effects of formaldehyde on pneumococcus antiserum, as well as those discussed in the following section, have been qualitatively reproduced with acetaldehyde, benzaldehyde and butyraldehyde. The first was almost as active as formaldehyde; benzaldehyde was only a fraction as active, and butyraldehyde was almost inert.

[8] Heidelberger and Kabat (15) have recently shown that the diazo-treated pneumococcus antibody also combines with carbohydrate. This we have been able to confirm. In the original paper of Eagle, Smith and Vickers (1) on the effect of diazo compounds, some evidence was presented against such combination; but as was there stated (page 629), the possibility of combination could not be excluded. The experiments of Heidelberger and Kabat clearly show that it does occur with diazo-treated antipneumococcus serum; and the present experiments further show that it occurs with formaldehyde-treated serum.

TABLE VI

The Effect of Formaldehyde Acting for 24 Hours on the Type II Precipitating, Agglutinating and Protective Action of a Mixed Type I and Type II Antipneumococcus Serum

(Serum treated as in first few columns of Table V)

Molar ratio of HCOH: protein	Approximate number of formolized NH_2 groups in antibody molecule per 100,000 molecular weight*	A. Precipitation of type specific polysaccharide: Varying amounts of treated serum + 0.2 cc. 1:400,000 acetylated SSS II	A. Supernatant + 0.2 cc. untreated serum (test for free carbohydrate)	B. Agglutination of bacteria (Type II): Varying amounts of treated serum + 0.2 cc. bacterial suspension: 4 hrs. at 37°C.	B. Readings after mild centrifugation	C. Protection of mice	Anaphylaxis experiments with guinea pigs sensitized to native horse serum. Amount of treated 1:2 serum injected: 2 cc.	1 cc.	½ cc.	Conclusions
0	—	4 4 3 2 1 ± 0	0 0 0 4 4 4 4	4 4 4 4 4 2 0	4 4 4 4 4 2 0	4SSSS 14SSS 234SS 234SS	D	D, D	D, D	Carbohydrate-precipitating activity of antiserum inhibited; combining affinity unaffected
7.5:1	3	± ± ± 0 0 0	Cl Cl Cl 4 4 4 4	3 4 4 4 0 0 0	4 4 4 4 4 0 0					
15:1	8	0 0 0 0 0 0	0 0 0 3 4 4 4	2 2 ± 0 0 0 0	4 4 4 4 4 0 0					
30:1	14		0 0 0 2 4 4 4	1 1 ± 0 0 0 0	4 4 4 4 2 0 0	34SSS 12234 2223S 12234			D, D	Agglutination inhibited; but antiserum can still combine with organisms, as shown by centrifuge agglutination, and can still combine with carbohydrate. Marked decrease in protective action
60:1	21		0 0 0 0 0 Cl 2	0 0 0 0 0 0 0	4 4 4 4 4 0 0					
120:1	27		—	0 0 0 0 0 0 0	4 4 4 4 2 0 0	224SS 11124 11234				
240:1	36		—	—	4 4 4 4 3 0 0					
480:1	41		—		4 4 4 3 1 0 0	12344 11111	Prostrated	Scratch		Progressive decrease, and eventual disappearance of combining affinity for carbohydrate, centrifuge agglutination and protective action. Activity of antiserum acting as antigen in guinea pigs sensitized to horse serum also impaired
960:1	43		—		3 3 3 ± 0 0 0		"	0		
1920:1	45		0 0 0 4 4 4 4		2 2 2 ± 0 0 0		0, 0			
3840:1	48		Cl 2 4 4 4 4 4		± ± ± ± 0 0 0		D, 0			
7680:1	49		2 4 4 4 4 4 4		0 0 0 0 0 0 0		0, scratch	Scratch	0	
15,360:1	50		4 4 4 4 4 4 4		0 0 0 0 0 0 0					

Cl = cloudy; numbers 1 to 4 represent increasing degrees of precipitation after 4 hours at 37°C., followed by 18 hours at 2°C.

* See †, Table I, and footnote 5, page 499.

In order to make a rough approximation of the number of groups in the antibody molecule affected by the HCOH, we may assume that serum protein has an average molecular weight of 100,000. If the molecular weight of the antibody is several times that quantity, as recent measurements by Heidelberger, Pedersen and Tiselius (12 *a*) indicate, the number of antibody groups affected is the corresponding multiple of the calculated number. It follows from the data of Tables V and VI that an amount of formaldehyde which could combine with at most 7 to 8 NH_2 groups in the antibody molecule for each 100,000 molecular weight (1 part 37 per cent HCOH to 2048 parts serum), which probably blocked no more than 3 to 4 such groups, and which did not demonstrably change the pH of the serum, nevertheless sufficed to destroy its precipitating activity with carbohydrate almost completely, without affecting its combining power with either the carbohydrate or the bacterial cell. An amount of HCOH which could combine with 15 NH_2 groups per 100,000 molecular weight, and which did combine with 9, inhibited spontaneous agglutinating activity, but again did not affect the combining power with bacteria, as shown by centrifuge agglutination. Eight to 32 times that quantity of HCOH was necessary before the combining power with either carbohydrate or bacteria began to be significantly impaired. This represents a concentration of 0.3 to 1.2 per cent HCOH, enough to block most of the NH_2 groups in the antibody molecule (sixth column of Table V). As in the case of other agglutinating antibodies (9), and of diphtheria antitoxin, it would therefore appear that free NH_2 groups are not primarily concerned in the combination between horse antipneumococcus serum and either the bacterial cell or the free carbohydrate. Paradoxically, concentrated refined antipneumococcus globulin was not affected by HCOH in concentrations which were found to destroy the aggregating activity of the native antiserum. This decreased susceptibility to HCOH of the isolated antibody is being further investigated.

One can only speculate as to whether the loss of flocculating activity with carbohydrate or bacteria caused by small concentrations of HCOH is due to the blocking of a few amino groups, or whether there is some more complicated reaction between the antibody protein and the formaldehyde. The successful reversal of the inactivated anti-

body by Chow and Geobel would indicate that the formation of a few $-N=CH_2$ groups is primarily responsible for the loss of flocculating activity (*cf.* page 502).

Some Observations on the Mechanism of Antigen-Antibody Aggregation

It was suggested in a previous communication (12 *b*) that the specific combining groups of antibody may be strongly hydrophilic, and that their elimination in the course of the antigen-antibody combination may result in a relatively insoluble compound. Antigen-antibody flocculation would simply reflect this decreased solubility. On this theory, only the combination of antigen and antibody is due to specific forces of attraction, and the secondary aggregation is non-specific. An alternative explanation of antigen-antibody aggregation has been suggested by Marrack (13) and Heidelberger (14). An elementary antigen-antibody compound would combine with similar compounds by virtue of residual specific linkages to form aggregates of increasing size, which eventually reach the limits of visibility. The antigen-antibody aggregate would accordingly be a lattice-like structure in which each molecule of antigen is bound to several molecules of antibody, and each molecule of antibody is similarly bound to several molecules of antigen. On this theory, both the first stage of combination and the second stage of aggregation are due to the same specific forces of attraction between antigen and antibody.

As shown in the present paper, an amount of HCOH sufficient to couple with only 7 or 8 groups of antibody for each 100,000 molecular weight, and which probably blocked no more than 3 to 4 groups, did not affect its combining affinity for the corresponding antigen, but completely inhibited the flocculating activity of antitoxin with toxin, and of antipneumococcus serum with carbohydrate. This finding is difficult to reconcile with the Marrack-Heidelberger theory of antigen-antibody aggregation. If aggregation were due to the same specific linkages which make for combination, as long as the antibody remains capable of combining with antigen, aggregation should follow as a matter of course; and the addition of a few molecules of formaldehyde should have no effect. Formaldehyde-treated (or diazo-treated) diphtheria antitoxin which combines with toxin should precipitate

at the unchanged optimum toxin: antitoxin ratio; and similarly treated pneumococcus antibody, which combines with carbohydrate,[8] should cause its precipitation. In both cases, the observed absence of visible aggregation is clearly not due to a loss of combining affinity, and cannot be explained on the Marrack-Heidelberger theory that formation of visible antigen-antibody compounds (agglutination and precipitation) is due solely to the specific combining groups.

Similarly, the fact that pneumococcus antibody adequately treated with either formaldehyde or diazo compounds fails to agglutinate pneumococci, despite the fact that combination has occurred (page 506), seems inconsistent with the mechanism of specific agglutination postulated by the investigators.[9]

The present observations are, however, consistent with the hypothesis that the specifically reactive groups of antibody protein contribute to its solubility, and that their elimination in the course of antigen-antibody combination results in a relatively insoluble antibody protein, and thus, in the precipitation of the antigen-antibody compound. One need only assume that formaldehyde (or diazo compounds) added on to antibody protein, most probably to the free NH_2 groups, increases its solubility. The following experiments were carried out to test that assumption.

Antipneumococcus antibody is normally water-insoluble and is precipitated from the antiserum on dilution with water. After treating serum for 24 hours at room temperature with as little as 1 part of 37 per cent HCOH to 2048 parts of serum there was a significant increase in the solubility of the antibody, as shown by a marked increase in the amount of water necessary to cause its immediate precipitation, and by a decreased amount of precipitate on dilution with ten volumes of cold water. This decrease was reflected both by the decreased agglutinating titer of the redissolved precipitate and by the actual amount of protein precipitated. Higher concentrations of HCOH resulted in an antibody which could no longer be precipitated

[9] Hooker (16) has recently presented evidence from an entirely different point of view which seems equally inconsistent with the theory that the secondary aggregation of antigen-antibody compounds is due to the same specific forces of attraction which bring about the original combination.

TABLE VII

The Effect of Formaldehyde, Acting for 24 Hours at 26°C., on the Water Solubility of Pneumococcus Antibody

...ti-...um	37 per cent HCOH	Approximate molar ratio of HCOH: protein*	Reactivity of whole treated antiserum (Same technic as indicated in Tables V and VI)								Amount of water necessary to cause beginning precipitation of treated antibody	Amount of antibody N precipitated after 2 hrs. at 2°C. on dilution with 20 cc. cold water	Conclusions
			Carbohydrate precipitation SSS II						Agglutination (Type I) Same amounts as previous section	Centrifuge agglutination (Tubes of preceding section)			
			0.2 cc.	0.1 cc.	0.05 cc.	0.025 cc.	0.0125 cc.	0.0062 cc.					
...c.	cc.										cc.	mg.	
.6	0	0	4	4	4	4	Cl†	0	444400	4444?0	3.3	2.5	Progressive increase in water solubility as antibody is treated with formaldehyde. Point at which antibody is no longer precipitable by water coincides with disappearance of precipitability by specific carbohydrate: Combining power with carbohydrate or bacteria unaffected
.6	0.0006	6:1	4	4	4	3	Cl	0	444400	444400	10.0	1.0	
.6	0.0008	8:1	4	4	4	Cl	Cl	0	444400	444400	20.0	0.9	
.6	0.0012	12:1	3	3	1	Cl	Cl	0	444400	444400	No immediate precipitation on dilution with water in any amount	0.4	
.6	0.0016	16:1	Cl	Cl	Cl	Cl	±	0	444300	444400		No significant amount of precipitate	
.6	0.0024	24:1	Cl	Cl	±	0	0	0	443200	444400			
.6	0.0032	32:1	0	0	0	0	0	0	333200	444400			
.6	0.0048	48:1	0	0	0	0	0	0	332100	444200			

* *Cf.* footnote 5, page 499; and Table I.

† Contents cloudy: no immediate precipitate.

by dilution with water or by dialysis (Table VII).[10] It is significant that the same amount of treatment which rendered the antibody water-soluble, also largely inhibited its precipitating activity with capsular carbohydrate (*cf.* Tables V, VI and VII). Similar experiments with diazo compounds have yielded qualitatively similar results. Wholly analogous to the increased solubility caused by formaldehyde and diazo compounds is the observation by Felton and Bailey (11 *b*) that horse antipneumococcus sera heated at 56°C. for 30 minutes in large measure lost their precipitating, agglutinating and complement fixing activity, but that their protective action *in vivo* was unaffected; and that such heated sera no longer yielded a precipitate on dilution with water.

These several observations with antipneumococcus and antitoxin serum strongly support the theory that antigen-antibody aggregation is primarily determined by the insolubility of the bound antibody. The formaldehyde-treated, diazo-treated or heated antibody can still combine with antigen, and specifically reactive water-soluble groups are thus eliminated. Normally, this would suffice to make the antibody protein sufficiently insoluble to cause visible flocculation of the antigen-antibody compound. In the treated antibody, however, the highly soluble groups formed by the addition of a few molecules of formaldehyde or of diazo compound to the antibody, groups which are not involved in its combination with antigen, apparently suffice to keep the compound in solution, and there is no aggregation.[11]

SUMMARY

Small amounts of formaldehyde inhibited the precipitating activity of horse diphtheria antitoxin with toxin and of horse antipneumococcus

[10] This amount of treatment with formaldehyde did not significantly affect either the pH of the solution, or the isoelectric point of the serum protein as determined by the optimum pH for precipitation. At that isoelectric point, however, there was a copious precipitate, no less than that obtained from untreated serum; indeed, strongly formolized serum yielded even more precipitate than the control, untreated serum.

[11] The fact that the antibody content of some antipneumococcus sera is lower when tested by carbohydrate precipitation than it is when tested by mouse protection or carbohydrate combination suggests that in these sera the antibody may be normally water-soluble to a greater extent than is usually the case (*cf.* 17).

serum with the homologous capsular carbohydrate. Approximately 1 part of commercial formaldehyde to 1000 parts of serum, acting for 24 hours, inhibited the flocculating activity completely. In both cases, the combining affinity of the treated antibody for the corresponding antigen was not demonstrably affected, as determined both by *in vitro* experiments and by animal protection. More intensive treatment of the antipneumococcus serum caused an apparent loss of its bacterial agglutinating activity, but on centrifugation the organisms cohered: combination had occurred, and only the spontaneous aggregation was prevented. These effects are the same as those previously described for diazo compounds, and have been qualitatively reproduced with acetaldehyde, benzaldehyde and butyraldehyde.

The quantitative relationships suggest that only a few groups in the antibody molecule need be modified by formaldehyde in order to prevent aggregation; and it is probable that these are some of the free NH_2 groups of the antibody protein. In marked contrast, the combining affinity of both antipneumococcus antibody and diphtheria antitoxin for the corresponding antigens was only slightly affected by amounts of formaldehyde which sufficed to block the free NH_2 groups rapidly and almost completely. Similarly, this amount of treatment did not affect the reactivity of these two antisera acting as antigen with a rabbit antiserum *versus* horse serum. The integrity of the NH_2 groups is apparently not essential for the activity of these sera acting either as antigen or as antibody; and the slow disappearance of their activity in concentrated HCOH is apparently to be ascribed to some secondary reaction other than the simple addition of HCOH to free NH_2 groups.

The present experiments do not support the theory that antigen-antibody aggregates are lattice-like structures built up from elementary antigen-antibody compounds because of residual specific combining groups. The aggregating activity of both antipneumococcus serum and diphtheria antitoxin was completely inhibited by procedures which did not demonstrably affect their combining power with antigen. This suggests that the aggregation of antigen-antibody compounds is a secondary, non-specific reaction. It is perhaps significant that the amount of formaldehyde which just sufficed to prevent aggregation also caused a marked increase in the solubility of the pneumococcus antibody, which could then no longer be precipitated

at serum pH by dilution with water or by dialysis. This strongly suggests that the loss of precipitating activity is actually due to the increased solubility of the antibody and supports the hypothesis that the primary cause of specific antigen-antibody aggregation is the relative insolubility of the bound antibody.

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THE DEGREE OF COMPENSATORY RENAL HYPERTROPHY FOLLOWING UNILATERAL NEPHRECTOMY

II. The Influence of the Protein Intake*

By LOIS L. MacKAY, Ph.D., T. ADDIS, M.D., and EATON M. MacKAY, M.D.

(*From the Department of Medicine of Stanford University Medical School, San Francisco*)

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The ingestion of a diet containing more than the usual amount of protein is followed by a remarkable increase in the weight of the kidneys (1–8). This investigation was designed to determine the influence of similar variations in the protein intake upon the degree of compensatory renal hypertrophy following unilateral nephrectomy.

Since the appearance of our first preliminary note on this subject (4) there have been two papers (9, 10) dealing with the influence of the protein intake upon compensatory renal hypertrophy in which the conclusions are quantitatively very much at variance with the data presented here. Since the difference between the results[1] of Smith and Moise (9) and Allen and Mann (10) and our own are due to their method of expressing results, it would seem desirable to obviate further confusion by defining more exactly what is meant by compensatory hypertrophy and to describe our method of measurement.

When, as in our experiments, one kidney is removed, the remaining kidney grows larger and it is this enlargement which is called compensatory hypertrophy. Compensatory hypertrophy is expressed as the percentage increase of the hypertrophied kidney over the probable weight of the same kidney at that time had the animal had two kid-

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[1] These investigators failed to include a control group treated in the same manner as their nephrectomized animals, so they were not actually measuring compensatory hypertrophy but rather the sum of the hypertrophy due to the increased protein intake plus the compensatory hypertrophy.

TABLE I

Experiment	Age		Protein in diet	Control group averages							Nephrectomy group averages							Kidney per 100 sq. cm. body surface			Compensatory renal hypertrophy
					Body weight							Body weight									
	Operation	Death		Number of rats	Initial	Gross at death	Corrected	Body surface	Kidney weight	Protein intake*	Number of rats	Initial	Gross at death	Corrected	Body surface	Kidney weight	Protein intake*	Control group	Nephrectomy group	Difference	
	days	*days*	*per cent*		*gm.*	*gm.*	*gm.*	*sq. cm.*	*mg.*	*gm.*		*gm.*	*gm.*	*gm.*	*sq. cm.*	*mg.*	*gm.*	*mg.*	*mg.*	*mg.*	*per cent*
1	30	70	12	18	40	169	163	338	633	0.45	17	31	161	154	325	896	0.47	187	261	74	39.6
2	30	70	18	25	42	179	170	347	644	0.61	26	42	173	164	340	903	0.59	185	266	81	43.8
3	30	70	31	18	47	216	212	403	835	0.95	17	51	220	216	404	1289	0.90	207	310	103	49.7
4	30	70	43	21	42	197	195	381	832	1.30	19	41	189	186	369	1235	1.40	221	335	114	51.6
5	30	70	67	29	39	167	159	332	964	2.49	29	40	157	149	318	1398	2.61	286	439	153	53.5
6	360	400	12	14	375	416	404	616	1024	0.19	11	356	391	385	654	1247	0.19	166	200	34	20.3
7	360	400	18	22	318	372	367	581	1035	0.38	19	325	365	360	573	1255	0.39	178	219	41	23.1
8	360	400	31	13	366	414	410	627	1102	0.56	14	354	382	378	593	1436	0.54	179	243	64	35.7
9	360	400	67	26	315	317	312	521	1145	1.34	27	325	306	308	516	1580	1.27	219	307	88	40.2

* Grams per 100 sq. cm. body surface per day. The average of the last 10 days of each experiment.

neys and been under identical conditions of environment, diet, and experimental variables of other kinds. Since it is impossible to weigh the kidney before and during the enlargement it is assumed that one kidney of a similar control animal equals the weight of the remaining kidney, had no compensatory hypertrophy occurred. Since control

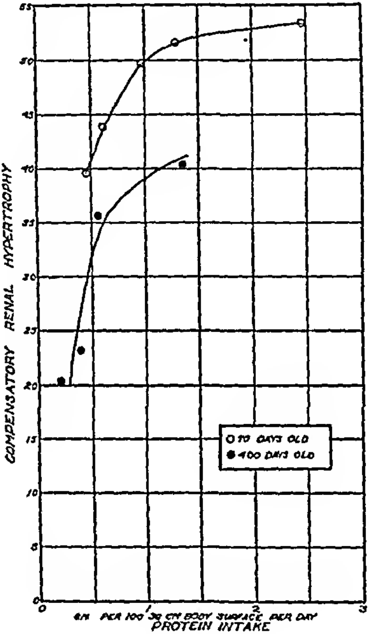

Fig. 1

and experimental animals of quite identical size cannot be obtained the actual comparison is made between the kidney weights per 100 sq. cm. of body surface (11). The degree of compensatory hypertrophy is measured 40 days after unilateral nephrectomy since after that time there is no further enlargement under our conditions (12).

The technical methods which were used have been detailed elsewhere (11, 12, 13). Diets containing 12.4, 18.0, 31.2, 42.9, and 67.2 per cent protein mostly as casein were used. The protein represented 10.5, 15.7, 27.3, 39.8, and 70.0 per cent of the calories respectively. These diets have already been described (8).

Our results have been summarized in Table I. In Fig. 1 the averages have been plotted and curves drawn to indicate the general relation between the protein intake and the degree of compensatory renal hypertrophy in these experiments. The lines in Fig. 2 suggest the relationship between the hypertrophied kidney weight and the protein intake at the two ages.

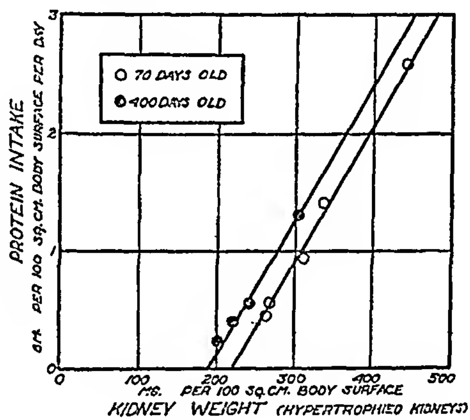

FIG. 2

From the data presented here a number of conclusions, governed by the conditions of our experiments, may be drawn. These are as follows:

1. In young rats and old rats an increase in the protein intake results in an increase in the degree of compensatory hypertrophy, which is of greater magnitude (Table I and Fig. 1) in old rats.

2. In young rats and old rats as the protein intake is successively increased above a minimum level the increment of increase in the degree of compensatory renal hypertrophy becomes less and less (Fig. 1). This decrease is more noticeable in young rats than in old rats but at both ages is particularly noticeable when the protein intake surpasses that which we have termed optimal (7) because it gave the

best body growth. Arithmetically this decrease is inevitable because the kidney weigbts of both two kidney rats (8) and rats with an hypertrophied kidney (Fig. 2) bear very nearly linear relationsbips to the protein intake.

3. Tbe protein intake has the same influence upon the kidney weight of rats with two kidneys at all ages (8) and the hypertrophic kidney is increased in weight the same absolute amount by a given increase in the protein intake in both old and young rats (Fig. 2). However, both at a zero protein intake or any given positive protein intake the hypertrophic kidney is larger the younger the organism. This results in the conclusion recorded in the first paper of this series (12) that compensatory hypertrophy of the kidney becomes less as age advances.

SUMMARY

Compensatory hypertrophy of the kidney in albino rats is increased by an increase in the protein intake. The effect is greater in old rats than young rats. Successive increases in the protein intake are followed by a reduction in the increase in the degree of compensatory renal bypertrophy.

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OBSERVATIONS ON THE PATHOLOGICAL CHANGES FOLLOWING EXPERIMENTAL HYPERTENSION PRODUCED BY CONSTRICTION OF THE RENAL ARTERY*

By CHARLES G. CHILD, M.D.

(*From the Department of Surgery of the New York Hospital and Cornell University Medical College, New York*)

PLATES 19 AND 20

(Received for publication, December 16, 1937)

In 1934, Goldblatt (1) described a method by which a persistent elevation of the systolic blood pressure could be produced in dogs by partial constriction of the renal artery. In this laboratory various experimental studies have been undertaken upon animals in which a hypertension of this nature has been induced and maintained for periods of time varying in length from 1 week to 10 months. As these animals have died or been killed in the course of the experiments, a post-mortem examination has been made of all the tissues which could reasonably have been expected to have been involved in this morbid process. It is the purpose of this report to record briefly the clinical picture manifested by these animals during life, and to consider the significant pathological changes which have been found after death, both on gross and microscopic examination.

Methods

Normal male and female dogs, varying in weight from 10 to 20 kilos have been used throughout the experiments. All blood pressure readings have been made by the carotid loop method of Van Leersum, and the normal level determined by daily observations for at least 1 month. Partial constriction of the renal artery has been secured by means of a small stainless steel clamp similar in design to that originally described by Goldblatt (1). Whenever animals have been killed a

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lethal dose of chloroform has been administered by inhalation. All the material for microscopic study has been fixed in 10 per cent formalin, imbedded in paraffin, and the sections stained with hematoxylin and eosin. When identification of connective tissue has been essential, Masson's trichrome stain has been employed.

Six animals have been studied which died within a few days after bilateral constriction of the renal arteries. These invariably presented a similar clinical picture; namely that of an overwhelming intoxication, the manifestations of which were a marked hypertension, vomiting, a bloody diarrhea, anuria, and terminally, convulsions and coma. The blood urea nitrogen was invariably elevated.

TABLE I

Animals Dying in Acute Hypertension Associated with Uremia

Experiment	Dog No.	Normal blood pressure	Hypertension	Duration*	Blood urea nitrogen at death	Remarks
		mm. Hg	*mm. Hg*	*days*	*mg. per 100 cc.*	
1	SA-36-62	140	270	7		Normal dog. Constriction of both renal arteries
2	SA-36-63	130	270	7		Normal dog. Died following constriction of both renal arteries for the second time
3	SA-37-72	150	270	7	44.0	Animal with but a single kidney and that transplanted to the femoral vessels. Died following constriction of the renal artery for the second time
4	SA-37-76	140	260	7	41.0	" "
5	SA-37-77	135	270	12	36.0	Animal with but a single kidney and that transplanted to the femoral vessels. This animal died following the third constriction of the renal artery
6	SA-37-84	140	210	4	76.0	Normal dog. Died following constriction of both renal arteries

* Time in days between constriction and death.

The Pathological Findings.—At autopsy gross examination uniformly revealed innumerable petechial hemorrhages, diffuse in distribution, though predominating in the cardiac musculature, and in the gastrointestinal tract. In several of the

animals these lesions were also found in the brain, both cortically and deep in the cerebral substance itself, in the skeletal muscles, the pancreas, the lungs, and superficially in all mucosa lined viscera. Microscopically, the source of hemorrhage was found to have been from the capillary bed. In the sections of all of the organs examined disintegration of the capillaries could be found in the center of small extravasations of erythrocytes. Particular attention was paid to the arterioles and walls of the larger arteries, but in these no variations from normal could be observed. In each case the kidneys were extensively damaged; in three the entire organ was infarcted, in two, merely a diffuse parenchymatous degeneration was observed, and in one both parenchymatous degeneration and multiple small infarcts were present.

These findings confirm those originally observed by Goldblatt (1) in animals in which too great a degree of constriction had been applied to the renal artery. In one of these animals (SA-37-77) a marked hypertension was produced by the third constriction of the blood supply to a single transplanted kidney. Shortly before death the animal suddenly became blind, and ophthalmoscopic examination revealed bilateral detachment of the retinae. On section of the globes this was found to have been a serous detachment, though the choroid itself was heavily infiltrated with fresh hemorrhage. No obvious etiology presented itself for this phenomenon, and it remains unexplained. Perhaps its chief interest lies in its comparison with animal SA-36-55 which suffered a similar accident in the course of a severe hypertension which had been present many months.

Twelve animals were killed in various stages of a persistent hypertension which had been present for from 1 to 10 months. In eleven of these no abnormalities were observed during life save those associated with secondary experimental procedures, for example pancreatectomy or destruction of the spinal cord.

The Pathological Findings.—At postmortem examination the gross findings were not striking, and no pathological changes were observed other than a slight diminution in the size of one or both kidneys. This apparent decrease in the size was not infrequently accompanied by thickening and pitting of the capsule due to scar tissue. The great vessels, particularly the aorta, were searched for evidence of atheromatosis as routine, but in no instance could such lesions be demonstrated. Occasionally the heart seemed enlarged, but this can be considered little more than an impression for no roentgenological measurements were made. On microscopic examination the pathological changes were confined to the kidneys and the arterioles. There was constant microscopic evidence of damage in one or both

kidneys quite similar to that originally described by Goldblatt (1). All degrees of hyalinization of the glomerular components have been found, as well as interstitial fibrosis, tubular degeneration, and thickening of the walls of the small arteries. In a few instances the glomerular tufts were diffusely adherent to their capsules, and crescent formation was not rare. One peculiar lesion was consistently present in the form of small foci of round cell infiltration composed of large and small lymphocytes and plasma cells. These were distributed indiscriminately in varying intensity throughout the cortex and medulla. A distinct attempt was made to discover animals, the kidneys of which showed definite thickening of the walls and diminution in the lumina of the arterioles without similar vascular changes elsewhere in the body. No such instance was found.

TABLE II

Chronic Hypertension

Experiment	Dog No.	Normal blood pressure	Hypertension	Duration*	Remarks
		mm. Hg	*mm. Hg*	*mos.*	
1	SA-36-9*a*	110	180	1.2	Died 1 day after destruction of spinal cord
2	SA-36-21	120	200	3.2	Killed 2 mos. after destruction of spinal cord
3	SA-36-31	110	200	2.8	Killed 1½ mos. after destruction of spinal cord
4	SA-36-36	115	190	3.3	Killed 1½ mos. after total pancreatectomy
5	SA-36-41	125	200	3.0	" " " " " "
6	SA-36-46	110	200	10.0	Normal control animal
7	SA-36-47	130	190	6.5	" " "
8	SA-36-54	120	200	1.1	" " "
9	SA-36-55	120	200–300	8.5	See below
10	SA-36-57	110	180	4.0	Normal control animal
11	SA-36-59	110	200	3.5	" " "
12	SA-36-64	150	220	1.5	" " "

* Time in months following constriction of the renal arteries.

As each organ was studied individually there was frequently found a definite degree of thickening of the arteriolar walls associated with decrease in the size of the lumina. This finding bore a constant relationship to the degree and duration of the hypertension. They have never been found in animals with only a mild elevation in the blood pressure, nor have they been associated with the severe form of the

disease unless it had been present at least several months. Further, the impression was distinct that, though these vascular lesions could be found in all of the organs, they were most pronounced in the cardiac and mesenteric vessels.

The twelfth animal in this group (SA-36-55) has seemed of sufficient interest to warrant consideration in some detail.

This was a young animal which manifested a marked hypertension for a period of 8 months. During the first 6 months of this time the blood pressure was consistently in the neighborhood of 200 mm. Hg, an elevation of some 80 mm. Hg above its normal level. During this first period nothing unusual was noted clinically. It survived uneventfully a reverse Eck fistula (3). This procedure which was followed in this animal by a transient rise in blood pressure lasting but a few days, has been shown to be without permanent effect in this type of hypertension. In the course, however, of the last 2 months of life the pressure slowly rose to over 300 mm. Hg. Associated with this the animal appeared vaguely unwell, ate but poorly, and became excited easily. A week before it was killed it suffered a transient hemiparesis lasting about 12 hours, followed within a few days by an ophthalmoscopically demonstrable detachment of both retinae. At no time was the blood urea nitrogen elevated above normal. The day the animal was killed it was 17 mg. per 100 cc. At autopsy there were few significant gross findings. The serous nature of the detachment of the retinae was evident on section of the globes, but in this case, as in that of Keyes and Goldblatt (4), there were also several partially organized retinal hemorrhages. One kidney appeared normal in the gross and microscopically save for definite arteriolar thickening, while the other apparently was diminished in size and its cortex thinner than usual, scarred and pale. On microscopic section it demonstrated all of the lesions which have been mentioned above (Fig. 1). Examination of the remaining organs failed to reveal any evidence of pathological change other than those of the arterioles associated with each tissue studied. These lesions were remarkable in their intensity and variation, and though they were most constantly present in the vessels of the heart and of the mesentery, they also were found in the brain, liver, pancreas, kidney, and skeletal musculature. Fundamentally, the picture presented was that of a necrotizing arteritis. As shown in Fig. 2 there is an acute verrucous arteritis with necrosis, hyaline thickening, periarterial round cell infiltration, and early periarterial fibrosis. In Fig. 3 a similar lesion is shown in the heart where thrombosis of the vessel has occurred causing an acute myocardial infarct. In Fig. 4 an apparently later stage in this disease is shown in which partial healing has occurred. Here there is a marked hyaline degeneration of the media, edema and fragmentation of the intima, and periarteriolar fibrosis. The marked diminution in the size of the lumen is quite evident. Fig. 5 shows a small artery in the liver in which the end point of this process has apparently been reached in that merely perivascular fibrosis, intimal thickening, and marked diminution in the lumen can be identified.

DISCUSSION

In the first group of experiments (acute hypertension) the interpretation of the findings has been most difficult. It has previously been pointed out (1) that such animals die in uremia, but the clinical picture aside from the hypertension is very different from that seen in bilaterally nephrectomized dogs. When both kidneys have been removed the animals appear little disturbed for several days and there is no hypertension; but, when both arteries (or the renal artery of a dog with but a single kidney) have been sufficiently constricted to cause death the symptoms of a severe intoxication associated with the rise in blood pressure appear in from 24 to 48 hours, and death may occur as early as the 4th day. Another interesting comparison has been drawn by Goldblatt (2). If both renal arteries are simultaneously ligated the clinical course follows more closely that observed in bilaterally nephrectomized dogs than in those subjected merely to severe constriction of the arteries. It has been pointed out that in experiments in which the arteries are completely occluded the hypertension is distinctly less than that developing after partial constriction (2). From such comparisons as these it is strongly suggested that the experimental conditions favoring the maximum hypertensive response are associated with a degree of intoxication greater than that attendant upon the uremia. Both on gross and microscopic examination the vascular lesions have been non-specific and throw little light on the mechanism whereby the arterial pressure is elevated; in fact, they might more logically serve to explain an hypotension. As intimated above they cannot entirely be due to the associated uremia. Though petechial hemorrhages are found in nephrectomized dogs which die in uremia, they are not so extensive nor do they appear so early as in these acutely hypertensive animals. The only conclusion which has seemed justified is that the ischemic kidney is capable of elaborating a substance, already hypothesized by Goldblatt (2), which, if the degree of constriction is too severe, is effective not only in elevating the arterial blood pressure, but also in profoundly affecting the capillary bed, and causing the death of the animal earlier than should be expected were uremia the only cause of death.

In the second group of experiments (chronic hypertension) the significance of these pathological changes is obscure. Obviously the

hypertension is renal in origin, and though at its initiation no pathological changes can be found by way of explanation, the arterial lesions which appear after it has been present for some time might well serve to explain its persistence. Certainly in the case of one of the animals which has been reviewed in detail, it is difficult to explain the further rise in blood pressure on any basis other than that of progressive arterial disease, apparently unassociated with any clinical evidence of renal failure.

SUMMARY

The clinical histories and pathological findings of six animals dying within a few days following bilateral constriction of the renal arteries have been presented. No explanation for the rise in blood pressure was found.

The clinical histories and pathological findings of twelve animals with a sustained hypertension have been considered together with the detailed account of one of these in which a marked degree of arterial disease was found. It has been suggested from the study of this group that, though no cause for the initiation of the hypertension was found, the induced arterial disease might account for its maintenance.

The author wishes to express bis gratitude to Dr. N. C. Foot of the Department of Surgery for his assistance in interpreting the microscopic material.

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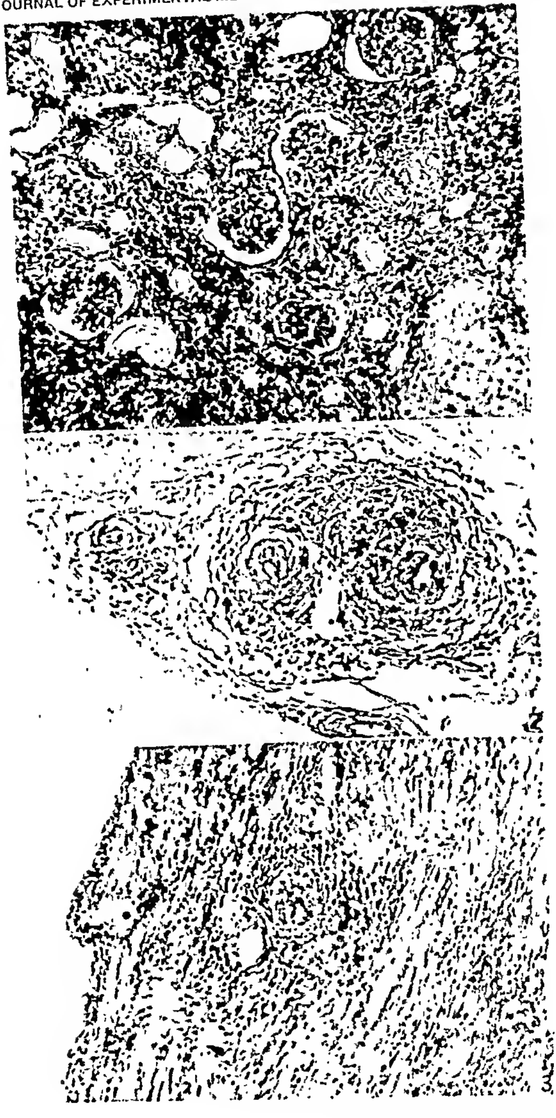
EXPLANATION OF PLATES

Plate 19

Fig. 1. Kidney showing hyalinization of Bowman's capsule, adhesion of the glomerular tuft to its capsule, interstitial fibrosis, tubular degeneration, round cell infiltration, and thickening of the walls of the arterioles. × 186.

Fig. 2. Acute verrucous arteritis. Mesenteric vessels. × 186.

Fig. 3. Acute arteritis with thrombosis and myocardial infarct. × 186.

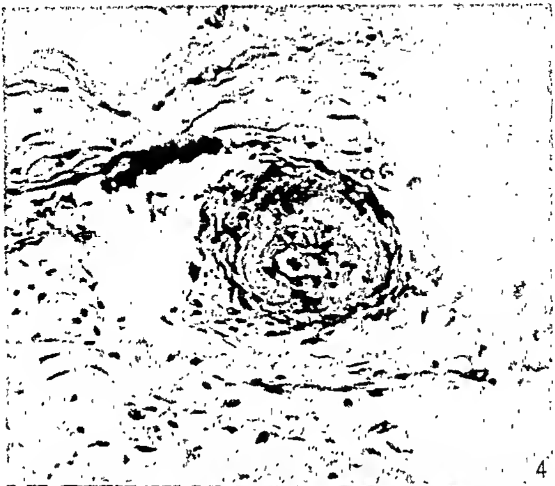


(Child: Hypertension from constricted renal artery)

PLATE 20

FIG. 4. Typical arterial lesion showing hyaline degeneration of the media, edema and fragmentation of the intima, diminution of the lumen, and periarterial fibrosis.

FIG. 5. Arteriole in the liver showing marked intimal thickening with all but complete obliteration of the lumen.



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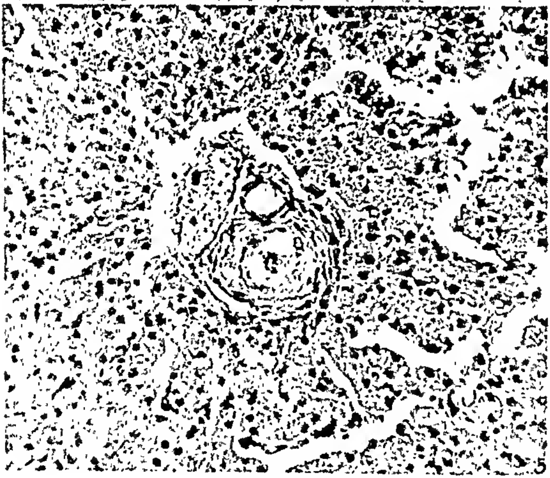

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(Child: Hypertension from constricted renal artery)

EVIDENCE OF ACTIVE IMMUNITY TO EXPERIMENTAL POLIOMYELITIS OBTAINED BY THE INTRANASAL ROUTE IN MACACUS RHESUS*

By S. D. KRAMER, M.D., L. H. GROSSMAN, and G. C. PARKER, M.D.

(From the Laboratory of the Infantile Paralysis Commission of the Long Island College of Medicine, Brooklyn, New York)

(Received for publication, November 19, 1937)

Epidemiologic and experimental studies would lead us to believe that most human beings who come in contact with the virus of poliomyelitis develop immunity, only the occasional susceptible individual succumbing to the paralytic disease. Events are very different in the experimental animal. Monkeys (*rhesus*) are essentially uniformly susceptible to experimental infection, and exposure to the virus either results in the experimental disease, or, provoking no response, leaves the animal susceptible to subsequent infection. So far as our knowledge goes, immunity develops in the monkey only through an attack of the experimental disease or by vaccination.

In a preliminary communication[1] we reported briefly on apparent successful alteration in susceptibility of *rhesus* monkeys to intranasal instillations of potent virus, and the subsequent development in most of the resistant animals of what appears to be an active immunity, as indicated by both the neutralization and intracerebral tests. We had, it appeared, succeeded in vaccinating the animals by the nasal route, to the best of our knowledge a hitherto unrecorded phenomenon. In the present paper we wish to present in detail the procedures and further results of the experiments which we believe are significant from epidemiologic and theoretical points of view, and which perhaps may have some practical bearing.

* This work was supported by a grant from the President's Birthday Ball Commission for Infantile Paralysis Research.

[1] Kramer, S. D., Grossman, L. H., and Parker, G. C., *Proc. Soc. Exp. Biol. and Med.*, 1936–37, 370.

Materials and Methods

Materials.—1. Surgical pituitrin (pituitrin S).

2. A mixture of adrenalin and ephedrine sulfate, in proportions of 1:10,000 of the former and 2 per cent of the latter, with 0.5 per cent chloretone as a preservative. This mixture is known commercially as adrephine.

3. A 5 per cent suspension of ground centrifugalized virus (Armstrong strain) was employed for intranasal infection. This strain proved to be potent; bringing down control animals in 7 to 9 days.

4. Our own laboratory strain, VM 11, was employed for neutralization and intracerebral tests, prepared as previously described[2] in large batches, and titrated every 2 or 3 months. The infective dose employed was two to ten times the minimal dose of virus necessary to bring down every animal in duplicate or triplicate titrations.

Procedure.—Healthy normal *rhesus* monkeys were selected. The animals were held in supine position with the head retracted; 0.5 cc. of pituitrin S was first instilled into each nostril by the dropper method; 5 minutes later 1 cc. of adrephine was dropped into each nostril. Following this treatment, and at stated intervals which varied in the different experiments, 1 cc. of a 5 per cent centrifugalized suspension of poliomyelitis virus (Armstrong strain) was instilled into each nostril.

Resistance to intranasal infection was determined by instillations of 1 cc. of a 5 per cent suspension of the potent Armstrong virus into each nostril daily for 3 successive days (intranasal test).

Neutralization tests were carried out in the manner previously described.[2] The animals were bled at varying intervals during the experimental period. 1 cc. of the undiluted serum to be tested was mixed with 1 cc. of virus, representing two infective doses (determined as described above) of a 5 per cent suspension of VM 11, in 1 cc. of saline. The mixture was incubated for 2 hours at 37°C., kept in the ice chest overnight, and 1.2 cc. of this mixture was inoculated intracerebrally into test animals.

The intracerebral test consisted of inoculating into the left or right frontal lobe of the experimental animal an infective dose of virus (in 1 cc. of saline).

All intranasal, neutralization, and intracerebral tests were accompanied by normal controls, to check the potency of the virus.

Protective Action of Pituitrin S and Adrephine (5 Days Preliminary Treatment)

Experiment 1.—Each of 7 animals received pituitrin S and adrephine as described above, every 12 hours for 5 days, or ten instillations in all. 12 hours after the last instillation, each animal received 1 cc. of the virus suspension into each nostril on 3 successive days. One animal died of dysentery. Of the 6 remaining

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[2] Kramer, S. D., Schaeffer, M., and Park, W. H., *J. Immunol.*, 1934, 27, 199.

animals, 2 succumbed, 4 resisted infection. Of the 4 surviving animals, one received a second series of three nasal instillations of virus 15 days after the first intranasal test. This animal died of tuberculosis 25 days later. A second intranasal test was done on one animal 75 days after the first nasal test, the remaining 2 animals were tested 100 days after the first intranasal test. All 3 animals survived. Neutralization tests performed on three serums obtained 18 days and one obtained 57 days after the first exposure to the virus, showed that 2 animals had developed neutralizing substance; two serums failed to neutralize. An intracerebral test performed on 2 animals 111 days and on one animal 86 days after the first intranasal test resulted in the experimental disease in all 3 animals; one animal developing a mild attack from which it recovered. Of the 14 controls used, all but one succumbed within the usual incubation period of the virus; the single survivor being one of the 4 used as intranasal controls.

Experiment 2.—Each of 6 animals was treated with pituitrin S and adrephine, for 5 days, in the same manner as the animals in Experiment 1. 24 hours after the last treatment, 2 of this group received the first of three daily doses of virus intranasally; 1 cc. into each nostril. 2 additional animals received the first of three daily doses 48 hours after the last treatment, and the last 2 animals received virus 96 hours after the last treatment. The 2 animals which were infected 24 hours after the last treatment resisted infection. Of the remainder, 2 succumbed to the disease, a third, No. 9-3, died of a rapidly progressing anemia, and the other died an accidental death. Neutralization tests were done on the serums of the 2 survivors, obtained 21 days after the last inoculation with virus; both serums neutralized. A second intranasal test was performed 43 days after the first series of intranasal instillations; both animals survived. Intracerebral tests were performed 56 days after the first intranasal test; one animal survived and one succumbed after a prolonged incubation period (27 days). All of the 11 controls used in this experiment succumbed to the disease.

Protective Action of Pituitrin S and Adrephine (7 Days of Treatment)

Experiment 3.—Six animals received two daily instillations of pituitrin S and adrephine 12 hours apart for 7 days. 24 hours after the last treatment (8th day), 2 of these animals received the first of three daily intranasal doses of virus; a second pair of animals received the first intranasal dose of virus 48 hours after the last treatment; and the last 2, 96 hours after the last treatment. Both animals infected 96 hours after the last treatment succumbed. One of the 2 animals infected 48 hours after the treatment succumbed and one survived. Both animals infected 24 hours after the last treatment survived. Neutralization tests were done on the serums of the 3 surviving animals, obtained 24 days after the third instillation of virus; two neutralized and one failed to do so. The animals were tested intranasally a second time 26 and 27 days after the first intranasal test; all survived. Intracerebral tests performed 40 to 41 days after the first intranasal test, resulted in the experimental disease in 2 of the 3 animals. All 10 controls succumbed.

Of the 19 animals included in these three experiments, 9 survived the intranasal test and 2 died from causes other than poliomyelitis. 6 of the group developed neutralizing substance in the blood serum, 2 of these 6 withstanding intracerebral inoculation. One animal succumbed to the intracerebral test after a prolonged incubation period (27 days), and a second succumbed to a mild attack from which it recovered. Of the 9 animals, all but one had received virus 12 to 24 hours after completion of the treatment with pituitrin S and adrephine; the ninth animal received virus 48 hours after the last treatment. The animals which had received virus after 96 hours, all succumbed. 34 of the 35 controls used in these 4 experiments developed the experimental disease.

Action of Pituitrin S and Adrephine in Combination with Virus

Experiment 4.—2 animals received daily instillations of both pituitrin S and adrephine, as well as virus. The pituitrin S and adrephine were instilled at 11 a.m. and the virus at 5 p.m. One of these animals succumbed to the disease in 11 days; the control having come down in 7 days. Daily treatment with pituitrin S and adrephine and instillations of virus was continued on the surviving animal for 28 days when it was discontinued, and on the 29th, 30th, and 31st days the animal received instillations of virus alone. The animal survived. Serum, obtained 29 days after the beginning of the experiment, failed to neutralize the virus. 9 days after the intranasal test, the animal was inoculated intracerebrally with an infective dose of a 5 per cent suspension of virus in 1 cc. of saline and survived. All 6 controls succumbed to the experimental disease.

Experiment 5.—4 animals received daily two treatments of pituitrin S and adrephine (at 9 a.m. and 5 p.m. respectively). At 1 p.m., or 4 hours after the first treatment, the animals received 1 cc. of a 5 per cent suspension of virus into each nostril. 2 of the group succumbed to the experimental disease in 9 days. The remaining animals survived 20 days of treatment and nasal instillations of virus, at which time the treatment and virus instillations were discontinued. They were tested by intranasal instillations of virus on the 21st, 22nd, and 23rd days; both survived. Neutralization tests were performed on the serums of these animals, obtained 20 days after the beginning of the experiment; both neutralized. Intracerebral tests were performed 9 days after the last intranasal test; both animals survived. All 5 controls employed in this experiment succumbed.

Experiment 6.—4 additional animals received the same daily treatments and exposures to virus as in Experiment 5. One animal succumbed in 9 days after the last inoculation of virus; 3 survived 22 days of treatment and exposure to virus. Neutralization tests were done on the serums obtained 22 days from the beginning of the experiment; one serum neutralized, one showed partial neutralization, and

one failed to neutralize. Intracerebral tests were done 23 days from the time the experiment was started; one animal succumbed to the experimental disease and 2 survived. The 4 controls employed in this experiment succumbed.

The survival of 5 animals in the above three experiments suggested the development not only of resistance to intranasal infection, but apparently of an active immunity, as indicated by their resistance to intracerebral inoculation and the appearance of neutralizing substance in the blood serums. These experiments were therefore repeated.

Experiments 7 and 8.—One group of 4 animals (Experiment 7), received daily for 31 days a single instillation of pituitrin S and adrephine at 11 a.m., followed by virus at 5 p.m. A second group of 4 animals (Experiment 8), received daily for 31 days two treatments with pituitrin S and adrephine at 9 a.m. and 5 p.m. respectively, combined with virus instilled at 1 p.m. Only one of the animals which had received a single treatment of pituitrin S and adrephine survived. That single animal, however, developed neutralizing substance in its serum 31 days after the beginning of the treatment, survived an intranasal test, and resisted the intracerebral test done 54 days from the beginning of the experiment. 3 of the 4 animals which had received two treatments and one instillation of virus survived; all 3 withstood an intranasal test and 2 resisted an intracerebral inoculation. Only one of the 3 animals developed demonstrable neutralizing substance, and that animal was one of the 2 that resisted the intracerebral test. All 22 controls in these two experiments succumbed.

Of the 18 animals used in these five experiments, which had received treatment with pituitrin S and adrephine in combination with virus instillations, 10 survived. 2 of these 10 survivors belonged to the group which were treated with pituitrin S and adrephine once a day, the remaining 8 twice a day. 5 of the 10 survivors developed neutralizing substance in their blood. 7 of the animals were tested intranasally and all survived. 8 of the group survived the intracerebral test. All of the 37 controls used in these five experiments succumbed to the experimental disease.

Action of Pituitrin S and Adrephine Administered in Combination with Virus Preceded by 5 Days Preliminary Treatment

Experiment 9.—4 animals received preliminary treatment with pituitrin S and adrephine once a day for 5 days. From the 6th day on and daily for 26 days they received instillations of pituitrin S and adrephine at 11 a.m., and of virus at 5 p.m. 3 animals succumbed in 8, 11, and 18 days respectively. The one animal which survived this procedure developed neutralizing substance 26 days after beginning

treatment and virus instillations. It resisted an intranasal test, and also intracerebral inoculation 54 days from the beginning of the experiment. 14 controls were used, all of which succumbed to the experimental disease.

Experiment 10.—4 animals received preliminary treatments with pituitrin S and adrephine, at 9 a.m. and 5 p.m. daily for 5 successive days. On the 6th day, instillations of virus (administered at 1 p.m.) were combined with the treatment and continued for 26 days. Of the 4 animals, one was found dead 17 days after the beginning of the experiment, but as indicated by histologic examination, not of poliomyelitis. The 3 remaining animals survived. All 3 developed neutralizing substance in their serums by the 26th day after the onset of the combined procedure, resisted an intranasal test, and also an intracerebral test performed 54 days from the beginning of the experiment. All 14 controls used in this experiment succumbed.

In the following two experiments a spray was employed in introducing the pituitrin S and adrephine, instead of the pipette as in the previous experiments.

Experiment 11.—6 animals received a single application a day of pituitrin S and adrephine by the spray method on 5 successive days, and beginning with the 6th day and daily for 24 days the applications of pituitrin S and adrephine were administered at 11 o'clock in the morning and virus dropped into the nostrils at 5 o'clock in the afternoon. Of the 6 animals, 5 succumbed, in from 9 to 23 days. The single survivor developed neutralizing substance 24 days after the onset of the combined treatment and virus instillations, resisted an intranasal test, and survived an intracerebral test performed 46 days from the onset of the experiment. The 7 controls used in this experiment succumbed.

Experiment 12.—8 animals received daily two treatments with pituitrin S and adrephine (at 9 a.m. and 5 p.m. respectively), instilled by spray on 5 successive days. Treatment twice daily with pituitrin S and adrephine in combination with a single daily instillation of virus at 1 p.m. was continued for 26 days. 7 of the 8 animals survived this procedure. 5 of the 7 animals developed neutralizing substance 26 days from the onset of the combined treatment and virus administration. 6 of the group resisted an intranasal test and also the intracerebral test performed 47 days from the beginning of the experiment. All of the 11 controls succumbed to the experimental disease.

Twenty-two animals were included in these four experiments. All received preliminary treatment with pituitrin S and adrephine for 5 successive days and this was followed by combined treatment and virus administration. 12 survived. Of this number 2 belonged to the group which had received treatment with pituitrin S and adrephine once a day; the remaining 10 animals had received two treatments a

day. 11 of the 12 animals survived intranasal and intracerebral tests. 10 developed neutralizing substance in their blood.

Protective Action of Adrephine

Experiment 13.—4 animals received 2 treatments with adrephine (at 9 a.m. and 5 p.m.), and virus at 1 p.m. 2 survived 37 combined daily treatments with adrephine and instillations of virus. Both of these animals developed neutralizing substance 37 days after the onset of the experiment; they both resisted an intranasal test and survived the intracerebral test, which was performed 59 days from the beginning of the experiment. All of the 9 controls succumbed within the incubation period of the disease.

Experiment 14.—3 animals received preliminary treatments with adrephine twice a day for 5 days. On the 6th day, 12 hours after the last treatment, the animals received the first of the 3 daily instillations for the intranasal test. One of the 3 animals was found dead 2 days after the virus was first administered (dysentery). The 2 remaining animals resisted the intranasal test; the blood serum, obtained 21 days from the day the intranasal test was started, neutralized the virus. Both animals resisted a second intranasal test done 21 days after the first, and both resisted intracerebral inoculation 33 days after the first intranasal test. All of the 7 controls succumbed to the experimental disease.

Protective Action of Pituitrin S

Experiment 15.—Three animals were treated twice a day (at 9 a.m. and 5 p.m.), with pituitrin S alone, and given one instillation of virus daily at 1 p.m. All 3 animals succumbed from 18 to 21 days after the beginning of the experiment.

The results of those three experiments seem to indicate that the protective action is largely due to the adrephine, the pituitrin S probably playing a secondary rôle. This is in accord with our original idea, since the pituitrin S was used to prolong the action of the adrephine.[3]

Comparable Experiments Using Solutions of Alum Sulfate-Picric Acid and Zinc Sulfate in Place of Pituitrin S and Adrephine

Solutions of Alum Sulfate-Picric Acid.—In the following three experiments an aqueous solution of 0.5 per cent each of alum sulfate and picric acid[4] was used in place of pituitrin S and adrephine.

[3] Suggested to us by Dr. Charles C. Haskell, chief toxicologist, Ciba Company.

[4] Armstrong, C., and Harrison, W. T., *Pub. Health Rep., U.S.P.H.S.*, 1936, 51, No. 33.

Experiment 16.—3 animals received preliminary instillations of a solution of alum sulfate-picric acid once a day for 5 successive days. On the 6th, 7th, and 8th days, the animals received nasal instillations of 5 per cent potent virus, 1 cc. into each nostril. 2 of the 3 animals survived. The serums of the surviving animals failed to neutralize the virus 24 days later; both survived a second intranasal test, 24 days after the first intranasal test, and both animals succumbed to the intracerebral test 47 days after the first intranasal test. All 6 controls succumbed to the experimental disease.

Experiment 17.—4 animals received preliminary nasal instillations once a day for 5 days with a solution of 0.5 per cent each of alum sulfate-picric acid, and from the 6th day on the animals received daily instillations of alum sulfate-picric acid at 11 a.m., and instillations of virus at 5 p.m. All 4 animals succumbed 7 to 21 days after the virus was started. The 4 controls succumbed within the incubation period of the virus.

Experiment 18.—4 animals received preliminary treatments with the alum sulfate-picric acid solution for 5 days. This was followed by daily intranasal instillations of virus alone. 3 of the group succumbed to frank attacks of the experimental disease, and the fourth was found dead on the 14th day, without having given any clinical signs or histologic evidence of poliomyelitis. The 4 controls succumbed.

The results of these experiments seem to indicate that solutions containing mixtures of alum sulfate and picric acid convey only partial protection to intranasal infection. There is no indication, furthermore, that prolonged exposure to virus alone, or in combination with solutions of alum sulfate and picric acid results in an active immunity.

Solutions of Zinc Sulfate.—In the following four experiments, 0.5 per cent solution of zinc sulfate in saline[5] was employed in place of pituitrin S and adrephine.

Experiment 19.—3 animals received daily for 5 days single instillations of zinc sulfate, and on the 6th day the first of the three daily instillations of virus. All 3 animals survived this first intranasal test; 2 animals succumbed to a second intranasal test 22 days later. The animal which survived failed to resist the intracerebral test performed 23 days after the second intranasal test. The serum of this animal did not neutralize the virus. The 6 control animals succumbed to the experimental disease.

Experiment 20.—4 animals received daily for 5 days single preliminary treatments with a solution of zinc sulfate and then single daily treatments with zinc

[5] Schultz, E. W., and Gebhardt, L. P., *Proc. Soc. Exp. Biol. and Med.*, 1937, 35, 524.

sulfate at 11 a.m., and instillations of virus at 5 p.m. 3 of the animals succumbed to the experimental disease; ooe survived 26 days of combined procedure of treatment and virus administration. The serum of the surviving animal, obtained 27 days after the virus was first given, neutralized but death by tuberculosis occurred a few days after the intracerebral test was done. 6 of the 7 controls succumbed; the one which failed to come down was one of the 4 animals used as intranasal controls.

Experiment 21.—2 additional animals treated in the same manner as the animals in Experiment 18, survived 30 days of treatment and exposure to virus. The serums of both animals failed to neutralize the virus and they succumbed to the intracerebral test performed 30 days from the beginniog of the combined treatment and administration of virus. The 3 normal controls succumbed.

Experiment 22.—4 animals received preliminary treatments of single daily instillations with the zinc sulfate solution for 5 days; this was followed by daily instillations of virus alone. 3 of the 4 animals survived 26 days; one succumhed in 6 days. Tbe serum of 2 of the survivors failed to neutralize the virus; while that of the third animal neutralized on the first test and failed when repeated. All 3 animals succumbed to the intraccrebral test given 33 days after the beginning of the experiment. The single animal, whose serum neutralized the virus on the first neutralization test, succumbed to the intracerebral test with an incubation period of 18 days. 6 of the 7 controls succumbed; the single survivor was one of the 4 animals used as intranasal controls.

It appears from these experiments that zinc sulfate offers effective protection against infection by the intranasal route for a period of about a month. However, these animals fail to give any definite evidence of the development of an active immunity, following prolonged daily exposure to the virus alone or in combination with zinc sulfate.

Experiment to Determine Presence or Absence of Delayed Protection of Pituitrin S and Adrephine

Experiment 23.—Each of 6 animals received two daily instillations 12 bours apart of pituitrin S and adrephine for 7 consecutive days. 2 weeks later 2 of these animals received the first of three daily intranasal instillations of 1 cc. of a 5 per cent suspensioo of virus into each nostril; the second 2 animals were similarly tested by intranasal instillations of virus 4 weeks after treatment with pituitrin S and adrephine; and the last 2 animals were tested 6 weeks after treatment. No delayed protective action was evident. 5 animals succumbed to the intraoasal test, and the sixth died of iotercurreot infection shortly after the intranasal test was started. Tbe 6 controls developed the experimental disease.

Protective Action of Subcutaneous Inoculations of Pituitrin S and Adrephine

In the following experiment the protective action against intranasal infection by pituitrin S and adrephine, when these substances are administered subcutaneously, was tested.

Experiment 24.—3 animals were inoculated subcutaneously with 1 cc. pituitrin S and 5 minutes later with 1 cc. adrephine. This procedure was repeated twice daily, at an interval of 8 hours (9 a.m. and 5 p.m.) for 5 consecutive days. On the 6th day these 3 animals and 2 controls all received the first of three instillations of the intranasal test. All of the animals succumbed, suggesting that subcutaneous injections of pituitrin S and adrephine afford no protection to intranasal infection.

Effect of Pituitrin S and Adrephine on Some of the Formed Blood Elements

In the following two experiments an attempt was made to determine if there were alterations in the type or total number of some of the formed blood elements following the administration of pituitrin S and adrephine by the intranasal and subcutaneous routes. The blood elements studied were total red and white cell counts and differential counts.

Experiment 25.—A group of 6 normal animals was selected and blood studies were done daily for 4 days to establish the degree of variation in these elements. On the next day and on the 4 succeeding (5 days in all), the animals received intranasal instillations of pituitrin S and adrephine twice daily, 12 hours apart (9 a.m. and 9 p.m.), and blood counts were done at 1 p.m. On the 6th day after beginning this treatment, the pituitrin S and adrephine were discontinued and all the animals received daily for 3 successive days single instillations of 5 per cent suspensions of virus (1 cc. into each nostril). The daily blood counts were continued for a period of about 2 weeks after the three doses of virus had been administered, or until the animals succumbed.

Experiment 26.—2 additional animals received daily for 7 days subcutaneous inoculations of increasing doses of adrephine. The initial dose of adrephine was 0.3 cc., and this was gradually increased so that the final dose was 1 cc. Blood counts were taken on these animals daily for the 7 days, approximately 4 hours after subcutaneous inoculation of the adrephine.

The total red blood cells of the 6 animals in Experiment 25, with one exception, remained relatively uniform throughout the study, this single animal dying of a severe progressive anemia. The white blood cells, however, showed frequent and marked variations, varying in one instance (monkey 9-1) from a minimum of

8,000 to a maximum of 30,000 per c.mm. without detectable cause. These variations occurred in haphazard fashion throughout all of the four phases of the experiment: the 4 days preliminary observation, the 5 days of treatment with pituitrin S and adrephine, the 3 days during which the virus was administered, and the final 12 day period of observation. This was true also of the differential counts particularly involving the adult segmented neutrophilic leucocytes, these frequently varying as much as 100 per cent without any appreciable associated changes in the total white counts. Eosinophiles appeared with considerable irregularity. During the 4 day preliminary period of observation, they were found but 3 times in the total of 24 counts. Two of the counts showed 1 per cent, the third 2 per cent. During the period of treatment with pituitrin S and adrephine, the eosinophiles were found 15 times out of a total of 30 counts, the percentage varying from 1 per cent to 4 per cent. During the 3 days when virus alone was administered, they were found 8 times in the 18 counts done. In the 34 blood counts done during the last 12 days of the study, eosinophiles were observed 13 times, varying from 1 per cent to 5 per cent of the total white count.

There appeared to be, therefore, a tendency for eosinophiles to appear more frequently when the pituitrin S and adrephine were administered, and to drop off slowly when these substances were discontinued. The figures, however, are small and no definite conclusion can be drawn.

The presence of eosinophiles appears to have some relationship to the occurrence of intestinal parasites in this experimental animal. Such infections are common in the monkey. Ova of the parasites are frequently found in the stool, and at autopsy small cysts containing round worms are encountered along the root of the mesentery. The animals used in Experiment 25 of the blood studies given above, were fresh stock recently imported from India. Blood counts done for 2 successive days on 6 animals which had been in the laboratory for a period of many months tended to show eosinophiles in their blood streams more frequently; of the 13 blood counts done on these 6 animals, from 1 per cent to 3 per cent eosinophiles were found 8 times, suggesting that old laboratory monkeys tend to show eosinophiles more frequently, possibly as a result of their intestinal infestations.

In the 2 animals which had received the adrephine by subcutaneous inoculation (Experiment 26), eosinophiles were found 7 times in the 14 counts done, the percentage varying from 1 per cent to 3 per cent. There appeared to be no correlation between the appearance of eosinophiles and the increasing doses of adrephine. The red blood cells in

these animals remained fairly uniform, whereas the white cells tended to show similar, though less marked variations than the other animals studied.

Actions of Pituitrin S and Adrephine and Their Component Substances on the Virus in Vitro

The following experiments were devised to determine the action of the components of pituitrin S and adrephine on the virus.

The commercial preparation known as adrephine is an acid (hydrochloric) solution containing adrenalin (1:10,000), and 2 per cent ephedrine sulfate with a pH of 4.0–4.2. The pH of the pituitrin S is about 3.4, due to the presence of acetic acid. Both solutions contain 0.5 per cent chloretone as a preservative. The following solutions were prepared: (*a*) hydrochloric acid, pH 4; (*b*) acetic acid, pH 3.4; (*c*) adrephine diluted with an equal amount of saline; (*d*) pituitrin S diluted with an equal amount of saline; (*e*) 0.5 per cent solution of chloretone.

Experiment 27.—To 1 cc. of each of these solutions, 0.1 cc. of normal monkey serum was added and the mixtures kept at room temperature for ½ hour. Two infective doses of virus in 1 cc. of saline were then added to each test tube, thoroughly shaken, placed in the incubator for 2 hours, and kept in the ice chest overnight. The following morning 1.2 cc. from each test tube was inoculated intracerebrally into test animals. 2 controls were given virus and saline. The test animal inoculated with the adrephine-virus mixture was the only one which survived, all the others, as well as both controls, developing the disease.

Experiment 28.—A mixture was prepared of equal parts of adrephine and a 10 per cent virus suspension (Armstrong strain). A portion of this mixture was permitted to stand at room temperature for 30 minutes, following which it was introduced into the nostrils of 2 normal monkeys (1 cc. into each nostril). The 2 control animals received 1 cc. of a 5 per cent suspension of virus into each nostril. The balance of the mixture of virus and adrephine was incubated for 2 hours at 37°C. and kept overnight in the ice chest. On the following morning 2 normal animals received intranasal instillations of this mixture (1 cc. into each nostril). 2 control animals received instillations of a 5 per cent suspension of virus which had been incubated for 2 hours and kept in the ice chest overnight. This procedure was repeated daily for 3 successive days.

The 2 animals which had received instillations of adrephine and virus kept at room temperature for 30 minutes, as well as the 2 controls which had received instillations of 5 per cent virus suspensions, succumbed to the disease within the incubation period of the virus. The 2 animals which had received instillations of the adrephine-virus mixture which had been incubated for 2 hours and kept overnight in an

ice chest failed to come down. The 2 corresponding controls succumbed to the experimental disease.

It would therefore appear that under the experimental conditions, adrephine possesses some viricidal properties, although this is evident only after incubation at 37°C. and prolonged contact with the virus.

Histologic Studies of the Mucous Membranes of Animals Treated with Pituitrin S and Adrephine

Preliminary studies of the nasal mucous membranes of 12 animals treated with pituitrin S and adrephine show a mild active inflammatory reaction 12 to 96 hours after treatment. There was hyperemia of the corium with local leucocytosis, margination, and exudation into the interstitial tissue. This appeared to be a fairly constant feature in all of the sections studied. Various types of leucocytes were found in varying numbers, and in several of the animals sacrificed 12 and 24 hours after the last treatment, eosinophiles were prominent both in the corium and migrating through the epithelial layer of the mucous membrane. There was no desquamation or necrosis and edema was not conspicuous. There was no fibrin lining the mucous layer. This preliminary histologic investigation has served to indicate that no severe inflammatory or degenerative effects are to be expected from instillations of these materials. A more detailed study of the changes in the mucous membrane is being made.

Résumé of the Findings

The results of the preliminary experiments indicated that protection against intranasal infection was obtained in almost two-thirds of the animals which had received instillations of pituitrin S and adrephine twice a day for 5 days, when the intranasal test was begun 12 to 24 hours after the last administration of pituitrin S and adrephine. 8 of the 11 animals which had received such treatment resisted two intranasal tests. Only one of the 3 animals which had received virus 48 hours after the last treatment of pituitrin S and adrephine survived. Of the 7 animals in these experiments which succumbed to the first intranasal test, 5 had been tested intranasally 48 to 96 hours after the treatment, and 2, 12 hours after treatment.

There was some indication in these preliminary experiments that

the first intranasal test gave rise to an active immunity in some instances, since of the 9 animals which had resisted the first intranasal test, 6 had developed neutralizing substance in their serums from 18 to 21 days after the virus had been administered; all of these 9 survivors resisted a second intranasal test, performed from 26 to 100 days after the first intranasal test, and 2 animals resisted the intracerebral test. A third animal came down with a prolonged incubation period of 27 days, and a fourth animal came down with a mild attack of the disease from which it recovered.

Eighteen animals received continued daily treatments with pituitrin S and adrephine in combination with daily instillations of virus. 6 of these 18 animals received one treatment a day, and 12 animals received two daily treatments. Of the 6 which had received a single treatment, 2 survived the intranasal test, one developed neutralizing substance in its serum, and both resisted an intracerebral test. Of the 12 animals which had received treatment twice a day, 8 survived; 4 of these developed neutralizing substance in their blood, and one serum partially neutralized the virus. 6 of the 8 resisted the intracerebral test.

Twenty-two additional animals received combined treatment and virus instillations, subsequent to 5 days preliminary treatment with pituitrin S and adrephine before administration of the virus was started. 10 of these 22 animals received one treatment with pituitrin S and adrephine a day, and 12 animals received two such treatments a day. Of the 10 animals which had received a single treatment in combination with virus, 2 survived the procedures. Both of these animals resisted intranasal infection; both developed neutralizing substance in their serums, and resisted the intracerebral test. Of the 12 animals which had received two treatments a day in combination with virus, 10 survived; 9 of the animals resisted the intranasal test, 8 developed neutralizing substance in their serums, and 9 resisted the intracerebral test.

In the two small experiments in which adrephine alone was used, the results were essentially the same as when pituitrin S and adrephine were employed. The 2 animals[6] which had received 5 days of treat-

[6] A third animal used in this experiment died from an intercurrent infection shortly after the experiment was started.

ment with adrephine twice a day and intranasal test begun 12 hours after the last treatment, survived; both animals developed neutralizing substance in their blood serum, resisted a second intranasal test, and both resisted an intracerebral test. Of the 4 animals which had received daily administration of adrephine alone in combination with virus, 2 survived the prolonged exposure to adrephine and virus. Both animals survived intranasal tests, developed neutralizing substance in their serums and survived the intracerebral test. There was little or no indication that pituitrin S when used alone afforded protection, since all 3 animals thus treated succumbed, though 2 of them lived for 21 days after the beginning of the experiment, and the third for 18 days.

Pituitrin S and adrephine administered subcutaneously afforded no protection against intranasal infection, nor did these substances appear to exert a delayed reaction when administered by the intranasal route since all of the animals tested intranasally, 2, 4, and 6 weeks after the 7 days treatment, succumbed.

There was some suggestion that the intranasal and subcutaneous administration of pituitrin S and adrephine resulted in a slight eosinophilic response in the blood stream, but this appeared to be transient and was not marked. A somewhat more pronounced local eosinophilic response, however, was noted in the mucous membranes of the animals which had received intranasal instillations of pituitrin S and adrephine. This was noted in animals sacrificed 12 and 24 hours after treatment. The eosinophiles were part of the mild general inflammatory reaction of the mucous membranes in all of the treated animals.

There was some indication that adrephine possessed some viricidal action, but this was evident only after incubation for 2 hours at 37°C., and prolonged contact with the virus.

In the seven comparable experiments in which solutions of alum sulfate-picric acid and zinc sulfate were employed in place of pituitrin S and adrephine, there was no indication of any alteration in the underlying susceptibility of the animals, as indicated by their failure to develop neutralizing substance in their blood and their inability to resist the intracerebral test. Most of the animals treated with zinc sulfate were protected against intranasal infection for about a month.

Animals treated with alum sulfate-picric acid showed a less constant protection against intranasal infection, than the zinc sulfate-treated animals. The survivors of both groups of animals succumbed to the intracerebral test.

SUMMARY

The experiments reported above indicate that the intranasal instillations of pituitrin S and adrephine, alter susceptibility in the *rhesus* monkey. One-half to two-thirds of the treated animals resisted intranasal infection, and, moreover, most of the resistant animals which had received combined treatment and virus developed active immunity, as indicated by the presence of neutralizing substance in their serums and by their ability to resist intracerebral infection. We have, it appears, not alone modified in some fashion the usual reaction of this animal to intranasal infection, but we have also successfully vaccinated these animals by the nasal route, so that the response in animals more nearly approaches what we believe to be the response in human beings.

We have no knowledge of the mechanism by means of which pituitrin S and adrephine produce this apparent alteration in susceptibility, but since the outcome of continued exposure to virus in most of the animals treated with these substances results in immunity, we believe that this offers a more hopeful approach toward the control of the disease.

CHEMICAL STUDIES ON BACTERIAL AGGLUTINATION

IV. Quantitative Data on Pneumococcus R (Dawson S)-Anti-R (S) Systems*

By MICHAEL HEIDELBERGER, Ph.D., and ELVIN A. KABAT, Ph.D.

(*From the Departments of Medicine and Biological Chemistry, College of Physicians and Surgeons, Columbia University, and the Presbyterian Hospital, New York*)

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Antisera produced in rabbits by injection of pneumococcus in the R phase, or S phase as it has been termed by Dawson (1), have been examined mainly for their protective power (2). Since this proved to be low against the virulent S phase (Dawson M) little more has been recorded. However, in a study of an absolute, quantitative method for the estimation of agglutinins (3) it was found that accurate results could be obtained with pneumococcus R (Dawson S) as well as with the M phase (3, 4). The behavior of a number of anti-R (S) sera was studied with suspensions of acid-killed, heat-killed, and formalinized R (S) pneumococci, and quantitative precipitin estimations were also made with pneumococcus C substance (5). Relationships brought to light by this study are recorded in the present paper.

EXPERIMENTAL

Stock pneumococcus (Pn) Type I strain 192 R (S) and Type II strain D 39 R (S) cultures were used.[1] 5 to 10 liter lots were grown in meat infusion broth and were centrifuged off and killed either by acidification to pH 4 (designated Pn AK below), by heating to 60°C. for 0.75 hour (HK), or by letting stand at room temperature overnight with 1 per cent formalin (FK). The cells were centrifuged, washed with saline until appreciable quantities of nitrogen could no longer be detected in the supernatant, and diluted with saline containing 1:10,000 merthiolate[2] until the nitrogen content equalled 0.10 to 0.15 mg. per ml. Rabbits were

* The work reported in this communication was carried out under the Harkness Research Fund of the Presbyterian Hospital.

[1] These cultures were kindly furnished by Dr. Martin H. Dawson of this department.

[2] Manufactured by Eli Lilly and Company, Indianapolis, Indiana.

injected intravenously four times weekly for a period of 4 weeks with 1 to 4 ml. of the suspensions and sera were obtained 5 to 7 days after the final injection. In some instances a second course was given (indicated by a subscript 2 after the rabbit number). Only Gram-positive suspensions were used for the injections and analyses.

Agglutinin active against the various suspensions was determined as in (3) by addition of an accurately measured volume of suspension, usually 2.0 ml. containing 0.40 to 0.60 mg. of N, to 0.5 to 1.0 ml. of antiserum at 0°, letting stand in the cold for 48 hrs. with occasional stirring, centrifuging in the cold,[3] washing twice with 3.0 ml. of chilled saline, and analyzing the residues for nitrogen by a modification of the micro Kjeldahl method. The increase in nitrogen over that in the

TABLE I

Absence of Type II Specific Polysaccharide in Pneumococcus II R (Dawson S), Strain D 39

	0.5 ml. Felton solution B 85, Type II, horse	
	+ 2.0 ml. Pn II S (M) for each absorption	+ 2.0 ml. Pn II R (S) for each absorption
	mg.	*mg.*
Antibody N pptd. in first absorption	0.47	0.20
" " " " second " *	0.20	0.03
" " " " third " *	0.05	0.00
Total antibody N pptd	0.72	0.23

* Corrected for aliquot portion of supernatant used.

volume of suspension used represented the agglutinin nitrogen removed in the first absorption. In all cases the analysis was repeated with aliquot portions of the supernatant from the first centrifugation, and any additional amounts of agglutinin nitrogen taken out were added to the first result, correcting for the aliquot taken. In some instances third, fourth, and fifth absorptions were necessary and even then sera were not always completely freed from agglutinins. Alkalinity of the sera was not the only factor involved in this slow removal of agglutinins from certain sera, but more consistent results could apparently be obtained after adjustment of pH to 6.5 to 7. It was usually found advisable to centrifuge all supernatants a second time in order to throw down any pneumococci carried along. The number of tubes and labor involved in doing this could be

[3] A refrigerated centrifuge manufactured by the International Equipment Company, Boston, Massachusetts, was used.

held to a minimum by adding the first and second washings (and preferably a third) successively to the tube in which the original supernatant had been recentrifuged, centrifuging again after each addition, and combining any residue with the main portion of agglutinated cells.

Anti-C was determined as in (6) by addition of a moderate excess of C substance, preferably prepared from a pneumococcus type different from that with which the rabbit furnishing the antiserum was injected. This was, however, immaterial if either the C used for the analysis or the culture used for immunization contained no type specific polysaccharide. The tubes were allowed to stand at 0°C. for 48 hours and nitrogen estimations were made on the washed precipitates. C substance nitrogen in the precipitates was deducted.

In Table I are given data on the absorption of Type II antipneumococcus Felton solution (7) with Pn II S (M) and Pn II R (S) suspensions. Absence of Type II specific polysaccharide in the R (S) suspension is shown by the removal of far less antibody than is taken out by Pn II M.

Analytical data on the amounts of agglutinin in the various antisera for the different types of Pn suspension are summarized in Table II. Estimations of anti-C precipitin in a number of the sera are also given. Data are also included on the amount of agglutinin nitrogen removed by several of the suspensions from a mixed Type I and II antipneumococcus horse serum.

DISCUSSION

In the second paper of this series (4) it was shown that the quantitative, absolute agglutinin method (3) provided a means for detection of the presence or absence of an antigenic component in a bacterial strain. This was illustrated by showing the absence of the specific polysaccharide of Type I pneumococcus (S I) in the R (S) strain used, and the presence of S III in an incompletely degraded Pn III R. The I R (S) strain employed in the present studies was the same as that used in the earlier work. Evidence for the absence of S II in the Pn II R strain used is given in Table I. It will be noted that antibody absorption with this strain comes to an end with the removal of only one-third as much antibody as is taken out by Pn II in the S (M) phase.

Analytical data on rabbit antisera to formalin-killed, heat-killed, and acid-killed Types I and II R (S) pneumococci are given in Table

II. It is apparent that the principal antibody in a number of these sera is anti-C, and that in such sera antibodies to pneumococcus cell proteins play a minor rôle. The anti-C content in the I R FK series

TABLE II

Antibody Removed from Antipneumococcus R (S) Rabbit Sera by Homologous and Heterologous Pn R (S) Suspensions and by C Substance

Rabbit serum No.	Pn antigen injected	Antibody nitrogen removed per ml. serum						
		by pneumococcus R (S)						by C
		Type I FK	Type I HK	Type I AK	Type II FK	Type II HK	Type II AK	
		mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
3.35	I R FK	0.60		0.46	0.57			>0.52*
3.37	"	0.40†		0.34				>0.32*
3.43	"	0.30†		0.28				0.23
4.48	I R HK		0.26			0.27		
3.30_1	I R AK	0.15		0.14				0.06
3.30_2	"	0.32†	0.38	0.39	0.49†?		0.35	0.07
3.31	"	0.18‡		0.13				>0.13*
3.42_1	"			0.13				>0.15*
3.42_2	"						0.38†	0.01‡
1.76	II R FK	0.70	0.30	0.26	1.23†	0.64	0.37	
3.39	"	0.16‡	0.27†		0.44†	0.70†?		
3.32	II R AK	0.38§	0.66†		0.48†	0.66†		0.02‡
Anti-Pn I, II horse serum								
702	I, II S (M)	0.39	0.30†		0.39	0.27‖		

FK = formalin-killed, HK = heat-killed, AK = acid-killed.

* Redeterminations after 3 years gave 0.49, 0.26, 0.05, 0.06, respectively, for these sera. Serum 3.37 gave 0.27 mg. N with C I, 0.25 mg. with C II. The original analysis was made with C III.

† Sera not entirely exhausted after 3 to 4 absorptions.

‡ Analyzed 3 years after other values were obtained.

§ Supernatant gave 0.23 mg. more antibody N in 2 absorptions with Pn I R HK.

‖ Supernatant gave 0.11 mg. more antibody N in 1 absorption with Pn I R FK.

was particularly high, but whether or not the formalinized suspension is unusually well adapted to the production of this antibody cannot be stated on the basis of the small experimental material.

It is shown in a footnote to the table that the amount of anti-C found in serum 3.37 was the same whether the C used as reagent was derived from Type I or Type II Pn. The quantitative data accordingly support the conclusion of Tillett, Goebel, and Avery (5) that the same C substance is produced throughout the pneumococcus group.

It will be noted that, in general, the acid-killed suspensions removed less antibody from the various sera than did the heat-killed or formalin-killed suspensions of the same type. This is somewhat unexpected, as the acid-killed suspensions were used both as antigens and absorbents in the hope that Pn, killed by the relatively mild exposure to buffer at pH 4, near the isoelectric points of the cell proteins, would prove the best of the series. Possibly the ease with which the AK cells become Gram-negative (*cf.* Dubos (8)) is a factor, although only Gram-positive AK suspensions were used for the immunizations and analyses.

In general, Types I and II Pn suspensions which had been treated in the same way removed the same amount of antibody from a given antiserum to Type I or Type II Pn R (S). Only in the two Pn II R FK antisera was there any evidence of type specificity. Since it has been shown (Table I) that the Pn II strain used contained no type specific polysaccharide, and that C I and C II precipitate the same amount of antibody, it is possible that formalin treatment of the Pn II R (S) cells stabilizes a minor type specific antigen. The Pn I R FK antisera showed no such type difference, but in these sera there was little antiprotein and almost all of the antibody was anti-C, which appears to be equally reactive with C I, C II, or C III. The data, therefore, support quantitatively the conclusion of Avery and his coworkers (9) that pneumococcus protein is the same throughout this group of microorganisms, except that in the Pn II R FK cells, at any rate, an additional minor type specific antigen is indicated.

SUMMARY

1. The quantitative, absolute method of agglutinin estimation is extended to the reaction between Types I and II pneumococcus R (S) suspensions prepared in various ways and antisera from rabbits injected with these cells.

2. Quantitative estimations of the amount of antibody to pneumococcus C substance are also given.

3. The significance of the data is discussed.

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THE COURSE OF VIRUS-INDUCED RABBIT PAPILLOMAS AS DETERMINED BY VIRUS, CELLS, AND HOST*

BY JOHN G. KIDD, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

PLATES 21 AND 22

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The lesions caused by viruses range in character from the acutely necrotizing to the continually proliferative. Little is known, however, of the relationship of these pathogenic agents to the cells they affect beyond the fact that they have what appears to be an obligatory association with the latter. The living cells, not the organism of which they are a part, function as the real hosts of a virus, protecting it from such antiviral principles as may circulate in the blood. In the light of these circumstances one can readily understand how recovery comes about from virus infections causing death of the cells, for with this occurrence protection ceases, and the virus is exposed to such antiviral forces as the host may have possessed or acquired during the disease. No such explanation will serve, however, to explain the observed retrogression of growths due to those viruses which cause the infected cells to multiply in series. Under such circumstances one might expect the pathological proliferation to go on inevitably until death of the animal ensued. This does not always happen, however. Virus-induced growths vary widely in their course and frequently they dwindle and disappear (chicken tumors, rahbit papillomas). The study reported here was carried out with a view to learning more of the respective parts played hy a virus, the cells it acts upon, and the individual host in determining the course of a virus-induced growth. Special attention has heen given to the phenomenon of retrogression. As material the rabhit papillomas caused by the Shope virus (1) were utilized, for the reason that they are sharply circum-

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scribed, autochthonous growths of neoplastic character (2), which can be directly measured and scrutinized; and because they are known to go on growing even though strong antiviral antibodies appear in quantity in the blood of rabbits bearing them (3).

Primary Influence of the Virus and of the Cellular Fabric upon Which It Acts

The early course of the growths produced by inoculation of the papilloma virus into scarified skin can be referred to the pathogenic activity and the concentration of the virus material engendering them, and to the character of the cellular fabric upon which this acts. The primary influence of the virus on the behavior of the papilloma can be likened to that of a shot fired into a medium of greater or less resistance. A highly pathogenic virus may cause papillomas to grow for a time in a relatively unfavorable milieu, just as a shot of great velocity may produce some effect on a resistant medium. A weak virus can give rise to progressive papillomas in highly susceptible hosts as a shot of low velocity can travel far if little resistance is offered. Some virus strains produce papillomas sooner than do others, and titration tests have shown that the incubation time is generally shortest (with an irreducible minimum of about 7 days) in the case of the strains of virus that prove effective in greatest dilution; as furthermore that these active strains produce more vigorous growths, when inoculated according to a standard technique, than do strains with longer incubation periods.

Papillomas due to one inoculum may run a highly various course, however, even in related domestic rabbits. In some individuals the virus causes fleshy, vigorous papillomas, while in others of the same age and weight it gives rise to dry-topped, shallow-based growths which retrogress. Certain differences in thickness, opacity, and succulence seem to mark the skins of some rabbits as more or less favorable to growth of the papilloma; but repeated attempts to ascertain the character of these differences by histological study, or by micrometric measurement of skin thickness during life, have thus far proved unsuccessful. Whatever the nature of the differences they are not specific, for comparative tests have shown that those skins in which

vigorous papillomatosis is engendered react with a more profuse epidermal proliferation to an intradermally injected solution of Scharlach R in olive oil, than do the skins which give rise to indolent or retrogressing papillomas. An illustrative experiment will be given.

Experiment 1.—6 adult, gray-brown, domestic rabbits weighing 2000 to 2500 gm. were utilized, in which tattoo papillomas from the same inoculum had run different courses. In 2 of the animals the papillomas were large and fleshy; they had enlarged rapidly and steadily during the 15 weeks since the virus inoculations. In 2 others small, shallow based, low, dry-topped growths had enlarged slowly; while in the remaining 2 the punctate papillomas had appeared somewhat late and enlarged very slowly, retrogressing completely 3 to 4 weeks after their appearance. The hair was clipped away from both sides of these rabbits and 0.1 cc. of a saturated solution of Scharlach R in olive oil was injected intradermally through a fine needle into 6 comparable sites on each side of every animal. The sites were reinjected with 0.1 cc. of the Scharlach R preparation 7 and 14 days later. When the results were appraised, 5 days after the last of the injections, notable differences were found. Reactive mounds had been produced in all of the animals, and in any one individual the mounds were almost identical in character. Those in the rabbits bearing fleshy papillomas consisted of large, fleshy, scarlet papules with sharp contours: they measured 10 to 16 mm. across and were elevated 6 to 9 mm. above the surrounding skin. In the rabbits in which the papillomas had retrogressed on the other hand, the mounds were lower and smaller (10 to 14 mm. across, elevated 3 to 7 mm.), much less fleshy, and they graded more gradually into the surrounding skin. In the 2 rabbits with indolent papillomas the mounds were intermediate as regards size, fleshiness, and color. Microscopic section of several of the various sorts, procured by operation on the 5th day after the last injection, bore out the gross findings. The injections had elicited a marked epidermal proliferation in the skin of the rabbits bearing fleshy papillomas (Fig. 1), whereas in the skin of those in which the growths had retrogressed the proliferation was far less marked (Fig. 2).

These findings have been borne out in several further experiments with a number of skin irritants (dibenzanthracene in olive oil and extracts of coal tar in olive oil, corn oil, cottonseed oil, cocoanut oil, palm oil, and paraffin oil) applied by injection and inunction. Generally speaking, skins which reacted markedly to one irritant did so to others, and in them the papilloma virus caused the most vigorous growths.

The Rôle of the Virus in the Phenomenon of Retrogression

In a first attempt to ascertain the rôle of the virus in the phenom-

enon of retrogression, a group of comparable domestic rabbits were inoculated at many separate situations with 3 strains of virus known to differ in primary pathogenicity.

Experiment 2.—10 normal adult gray-brown rabbits weighing between 2000 and 3350 gm. were secured from a single dealer. All were inoculated at 4 points on each side with 3 different strains of virus. The first material (virus fluid A) was made from the naturally occurring warts of a cottontail rabbit trapped in East Texas.[1] These had been in 50 per cent glycerol for 6 weeks. Representative portions of the glycerolated warts were weighed, washed briefly in three changes of Tyrode's solution, and ground with sand. Sufficient Tyrode's solution was then added to make a 10 per cent extract, the extract was centrifugalized lightly, and the supernatant fluid was pipetted off for use. Two more 10 per cent extracts were prepared identically from material generously furnished by Dr. Shope,—virus fluid B from the natural warts of a Kansas cottontail (W.R. 1240), in glycerol for 8 months, and virus fluid C from those of a second Kansas cottontail (W.R. 1211), in glycerol for 10 months. The inoculations were made with an electric tattoo machine fitted with nine small sewing needles. A small drop of virus fluid was placed at each of several sites on the shaved skin and uniformly tattooed into an area about 2 mm. across. Immediately after the tattooing, the excess of virus was blotted up with a sterile sponge. 4 such inoculations were made with each virus fluid along horizontal lines on each side of the rabbits. The inocula were thus placed 3 to 4 cm. from one another, and their relative positions were systematically varied from rabbit to rabbit, so that all were exposed to the same influences of situation. A single virus fluid was inoculated into every rabbit before the next was proceeded to, and the tattoo needles were sterilized before each material was utilized.

The growths resulting from these inoculations were charted at intervals of 2 to 6 days. Chart 1 gives a summary of the findings. From this it will be seen that all 3 strains of virus caused papillomas in every inoculated rabbit, but that strain A was far less effectual than were strains B and C. Host susceptibility found general expression, the rabbits most susceptible to one virus strain being most susceptible to the others as well.

In 3 of the 10 rabbits (50, 51, 44) all the papillomas, of whatever derivation, enlarged steadily throughout the many weeks of observation; and it is noteworthy that all the growths on any one animal eventually attained a fairly uniform size, regardless of their virus derivation or differing primary course. In 6 animals, on the other hand, retrogression of all the growths took place simultaneously after a time; and the chart shows that in three of these cases (49, 47, 43) the enlargement had been as great during the early weeks as in the rabbits (50, 44) in which the papillomas grew progressively. In the case of D.R. 48, all of the growths began

[1] Secured through the endeavor of Mr. R. C. Adams.

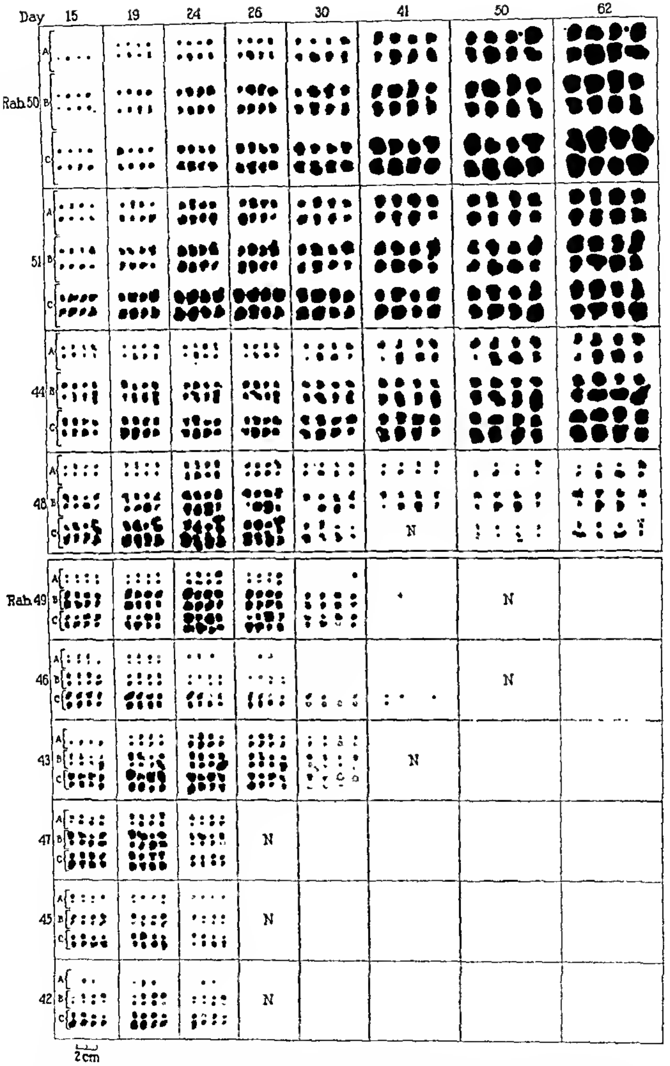

CHART 1. Comparative course of the papillomas induced by 3 strains of virus (A, B, C) in 10 domestic rabbits (Experiment 2).

to dwindle between the 26th and 30th days, and those from one strain of virus,—that which had given rise primarily to the most rapidly enlarging growths,—actually disappeared for a while, only to recur later. Plainly, in this instance, some intercurrent, generalized resistance of host derivation transiently influenced the course of all of the papillomas, though to an unequal extent,—the most rapidly proliferating cells being evidently most susceptible to its action. This phenomenon of transient intercurrent retrogression will be discussed more fully in a later section.

In this experiment retrogression of the papilloma was not due to peculiarities of the virus strains employed, but was consequent on some generalized influence exerted by the host; for, as a rule, all of the growths on any one animal enlarged or retrogressed together, even when engendered by virus strains of widely various pathogenicity.

The Recovery of Virus from Retrogressing Papillomas

What can be the nature of the generalized host influence responsible for retrogression of the papillomas in instances such as the foregoing? Mention has already been made of the fact that the sera of rabbits bearing the papilloma develop the capacity to neutralize free virus *in vitro* or on reinoculation. Previous experiments have demonstrated that the circulating, virus-neutralizing antibodies do not perceptibly influence the course of the growths in domestic rabbits, papillomas enlarging as rapidly in such rabbits when the blood has great antiviral potency as when this is slight (3). The living papilloma cells evidently protect the virus from the action of humoral antiviral principles, as other cells do other viruses (4). Because of this protection it should be possible to recover active virus from retrogressing papillomas, if it has not become attenuated in some other way.

In the attempt to recover the virus from retrogressing papillomas recourse was had to wild cottontail rabbits, for from experimentally induced growths in these natural hosts active virus can be procured frequently, instead of rarely as in domestic rabbits. The gross and microscopic manifestations of retrogression are similar in the two species, as many instances have shown. Furthermore, in the cottontail retrogression of the papilloma is the result of a generalized process, just as in the domestic species, and multiple growths induced by differ-

ent strains of the virus retrogress simultaneously. The following experiment demonstrates that active virus can be recovered from retrogressing papillomas.

Experiment 3.—10 "normal" cottontail rabbits from Kansas were inoculated broadcast on both scarified flanks with a highly active virus fluid (5 per cent suspension of the glycerolated papillomas of W.R. 1211), prepared as previously described. Growths appeared after about 10 to 15 days, but in 8 animals they were small, discrete, low, dry warts, and in several they soon retrogressed. Exuberant growths appeared, however, in 2 rabbits. One of these was killed on the 28th day and its growths preserved in glycerol; the other (W.R. 27-N) was ob-

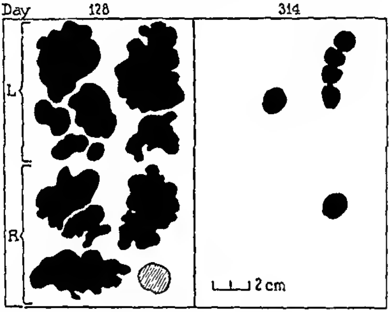

CHART 2. Retrogression and disappearance of the papillomas of W.R. 27-N (Experiment 3).

The hatched area indicates the outline of a subepidermal cyst near the superficial areas of confluent papillomatosis, such as is often seen about growths of exceptional vigor in cottontails. Virus was recovered in large amount from the small, dwindling papillomas present on the 314th day.

served over a long period. Its growths enlarged rapidly and progressively during the first 4 months to form characteristic, large, confluent and semiconfluent, papillomatous masses, 3 to 5 cm across and 1 to 2 cm. high, with cystic extensions into the connective tissue beneath and around them, of the sort forming when cottontail growths have exceptional vigor (5). During the subsequent months, nevertheless, they dwindled notably and most of them disappeared, as did all of the subcutaneous cysts. Tracings made on the 128th and 314th days, which illustrate the changes, are given in Chart 2. On the latter day, when the animal was killed, there remained on the left side 5 dry, pigmented, shallow, onion-shaped papillomas with constricted bases, none over 1.3 cm. in diameter; and a single, similar growth on the right side. These 6 small growths were excised

aseptically and put into sterile 50 per cent glycerol-Locke's solution in the refrigerator.

10 months later part of the glycerolated material was weighed, washed briefly in Tyrode's solution, ground with sand and made up to a 5 per cent suspension in Tyrode, and tested for infectiousness, along with 20 other suspensions made from glycerolated papillomas from as many cottontails in which growth had been progressive. All were inoculated into squares of skin on each of 3 normal domestic rabbits, according to a standard technique (3). By the 15th day thereafter, multiple, discrete growths had appeared on 2 of the rabbits where the W.R. 27-N suspension had been inoculated, and by the 19th day on the third rabbit as well. The growths due to this virus enlarged steadily into wholly characteristic, confluent and semiconfluent, fleshy, papillomatous masses, and they showed no tendency to retrogress. Upon comparison with the results from the 20 other materials it was evident that the W.R 27-N virus was notably pathogenic, the growths caused by it being even more vigorous than were those caused by most of the inocula which had been derived from enlarging papillomas. A second 5 per cent virus fluid prepared a few weeks later from portions of the glycerolated W.R. 27-N papillomas again produced large, characteristic growths when inoculated into 3 new test rabbits. The serum of W.R. 27 N, which had been secured by cardiac puncture on the day the papillomas were obtained, was found to neutralize completely a 5 per cent suspension of highly active virus (W.R. 1240,—more than 2000 infective units per dose) when mixed with it in equal parts *in vitro* and incubated at 37°C. for 2 hours prior to inoculation.

A later experiment yielded similar results. Another retrogressing papilloma from a second cottontail (W.R. 21-N) had become available for test,—namely a small, discrete, dry growth 0.8 cm. in diameter, secured on the 243rd day after inoculation. On the 116th day the papilloma had been 2.5 cm. in diameter, fleshy, truncated and onion-shaped, and it had undergone the reduction to its later size during the intervening period, while a number of other large papillomas produced on the animal by the same inoculum were retrogressing completely. A 5 per cent extract in Tyrode derived from it produced semiconfluent and discrete papillomas by the 15th day after inoculation in 2 of 4 domestic rabbits, and by the 20th day confluent and semiconfluent growths in all.

It is plain from these instances that the virus can persist in a highly pathogenic state in papillomas that have long been retrogressing, even though virus-neutralizing antibodies of high potency are circulating in the blood of the host. The implications of these facts will be discussed in a later section.

The Rôle of the Proliferating Cells

The evidence thus far given makes plain the fact that retrogression

of the rabbit papilloma is consequent on some general influence of host derivation, but it does not suffice to disclose the nature of this influence. Certainly retrogression can take place despite the continued presence of active virus in association with papilloma cells. This being so, one can scarcely suppose the process to be due to the antiviral forces of the host. Indeed everything indicates that the cells completely protect the virus against these forces.

The possibility suggests itself that the virus-infected cells may on occasion call forth a host resistance directed against themselves. To test this possibility an experiment was done in which the amount of papillomatous tissue was greatly varied in 3 groups of animals, and the incidence and course of retrogression were followed.

Experiment 4.—30 normal, adult, gray-brown domestic rabbits were secured from a single dealer, weighed, and notations made on the texture and thickness of their skins. The animals were then divided into 3 groups of 10, each group comparable in weight and skin characteristics. One group received on each side 32 identical tattoo inoculations of 10 per cent W.R. 1240 virus; the second group received 12 such inoculations, while the last group received only 2 on each side. At each site the tattooing was done over an area about 2 mm. across until a slight amount of blood came out into the drop of virus; which was then blotted off with a sterile sponge. The inocula were placed at points at least 2 cm. from one another.

As in the previous experiment all of the rabbits proved primarily susceptible to the action of the virus, growths appearing at almost every inoculation site, and nowhere else. There was considerable variation from host to host, but the growths on any one animal were of the same order of magnitude and fairly uniform as regards fleshiness and pigmentation. They were charted at intervals of 2 to 7 days until the 53rd day, and at longer intervals thereafter. The findings are summarized in Charts 3, 4, and 5. One of the animals died 3 days after the virus inoculation and hence is not represented in the charts. The early findings showed the 3 groups to be comparable as regards primary host susceptibility, and the incidence of retrogression did not vary significantly in the 3 groups.

Chart 3 shows the growths in 10 animals from the 3 groups in which the papillomas grew progressively, and in an eleventh in which a dubious late dwindling occurred. The findings are given for comparison with those next to be considered, and they need but little discussion. It will be observed that the papillomas showed a slight tendency to become larger eventually in the animals bearing fewer growths; but differences in the blood supply available to each growth, when there were 2, 12, or 32 on a side, respectively, may account for this difference, and crowding may have had some influence when many growths were present.

Chart 4 shows the course of events in 13 animals of the 3 groups, in which complete retrogression eventually took place. While the incidence of retrogres-

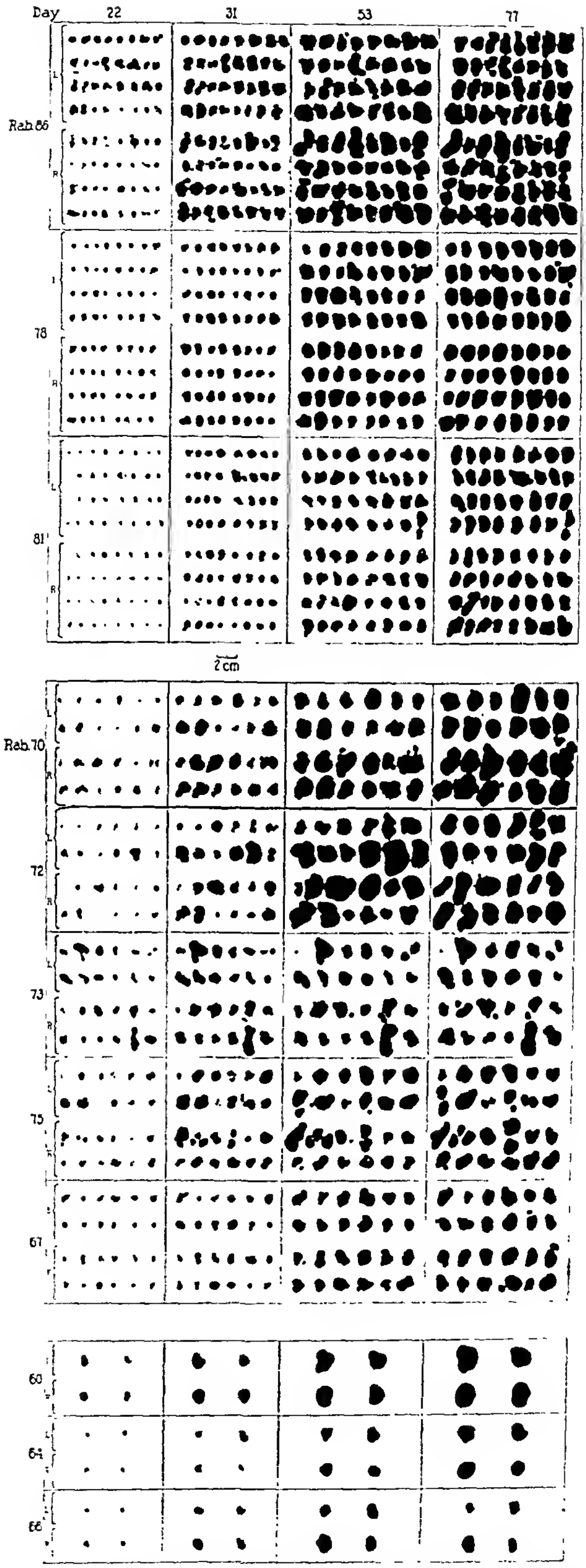

CHART 3. The progressing papillomas of 11 rabbits in Experiment 4. L, R = left and right sides respectively.

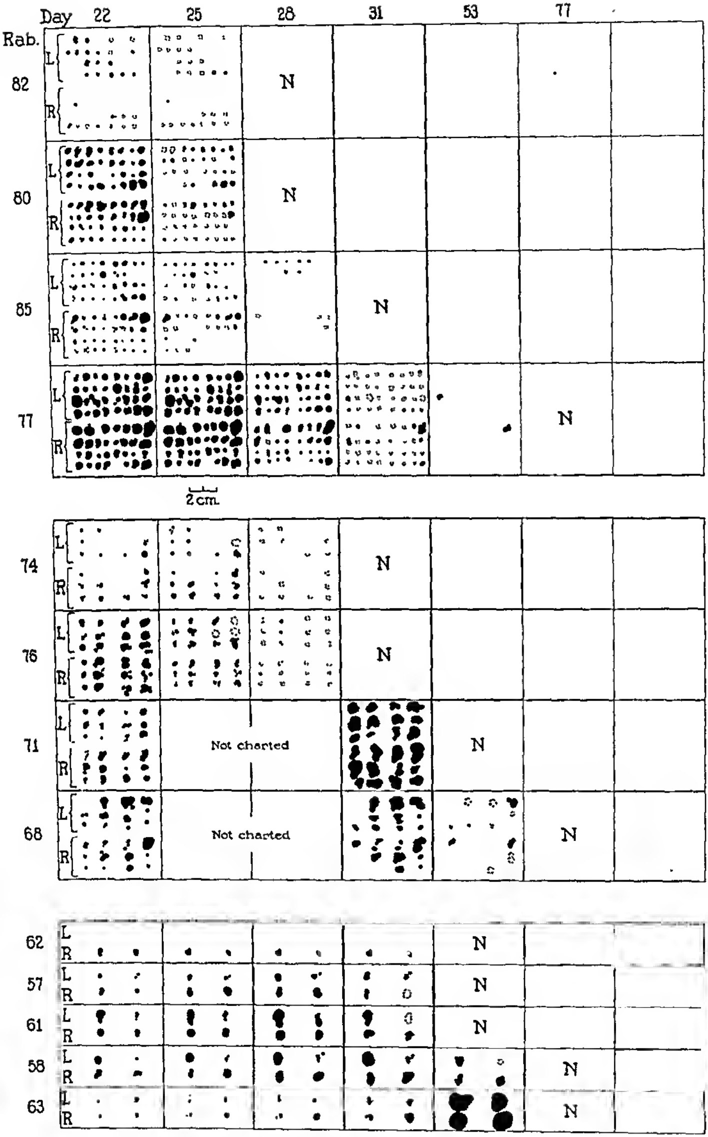

CHART 4. In the rabbits bearing many small growths the papillomas retrogressed earlier and more swiftly than in the others with few (Experiment 4). L, R = left and right sides respectively.

sion did not vary significantly from group to group, its time of onset and course did so markedly, as the chart shows. It will be seen that retrogression tended to begin sooner, and to take place more swiftly, the larger the number of papillomas present on a host, although the individual growths did not differ notably in size. In the 4 animals with 64 papillomas retrogression began earlier and was com-

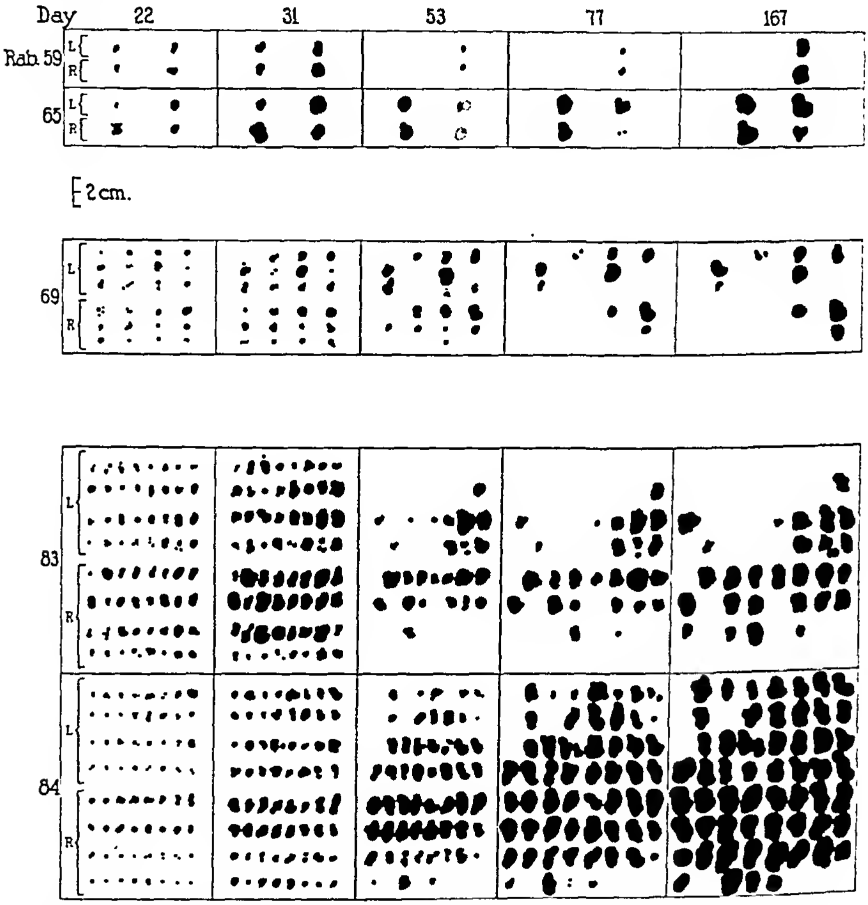

CHART 5. Transient, intercurrent retrogression of the papillomas in 5 domestic rabbits of Experiment 4. L, R = left and right sides respectively.

pleted more abruptly than in the animals bearing 24 papillomas. In these, in turn, retrogression took place earlier and more swiftly than in the rabbits with only 4 papillomas.

In the remaining 5 animals of the experiment a transient, intercurrent tendency to retrogression was evident. The findings (Chart 5) are comparable with those

in rabbit 48 of Chart 1. In one or two animals of each group some of the papillomas (usually the smaller) disappeared completely and permanently between the 31st and 53rd days, while concurrently other growths either disappeared transiently, dwindled somewhat, remained the same size, or, in rare instances, enlarged slightly. When all of the persisting papillomas began to grow again, some, which had disappeared leaving mere small, dry, shallow, scurfy thickenings in their places, reappeared as characteristic papillomas and enlarged steadily like the rest.

The influence of some generalized host resistance acting to bring about retrogression is clearly to be seen in the results of this experiment, as in Experiment 2 already discussed. In the present instance it was manifest in about the same number of individuals of each group, but to an unequal degree. Often the resistance was only transiently and feebly effective. The greater the number of papillomas induced, which is to say, the greater the total amount of proliferating epithelial tissue, the sooner did retrogression occur in the hosts predisposed thereto, and the more rapidly was the process consummated. Since the influence of virus-induced antibodies can be ruled out as responsible for the course of events, there is reason to suppose that the host resistance was directed against the cells themselves; and there is the more ground for thinking so because the manifestation of this resistance varied directly, both in time of appearance and in effectiveness, with the amount of papilloma tissue present.

The Histological Changes during Retrogression

The histological findings where papillomas are retrogressing constitute significant evidence of a host resistance directed against the proliferating cells as such, for they are essentially identical with those observed where transplanted tissues (neoplastic or non-neoplastic) retrogress as a consequence of resistance known to be directed against the cells.

The microscopic changes associated with retrogression of the rabbit papilloma have already been described, and the resemblance noted to those about retrogressing tissues generally (2). In a further study of the matter, sections of a number of small, discrete, retrogressing growths stained with eosin and methylene blue have been examined. The results extend the previous findings.

Much of the retrogression appears to be due to a slower rate of cellular proliferation, with result that differentiation outstrips it and death of the cells occurs through maturation into keratinized scales. It is often possible to find areas where

this process is more advanced than elsewhere; and in these the cells are stained less darkly, and mitoses are less frequently seen, while lymphocytes and makrophages are often present in considerable number in the underlying connective tissue. Everywhere the living papillae become progressively thinner, the epithelial layer more shallow, and finally there is left only a smooth scar covered by epidermis somewhat thicker than the normal, but appearing to be merely hyperplastic. The whole process suggests a gradual waning of cellular activity under the influences of an unfavorable environment, with eventual death by maturation of all the remaining virus-infected cells.

Attempts to Elicit Resistance Directed against the Papilloma Cells

In view of the evidence indicating that retrogression of rabbit papillomas may be due to a generalized host resistance elicited by and directed against the papilloma cells as such, it seemed well to make a direct attempt to bring about such resistance experimentally. In the case of transplantable mouse and rat tumors this has been accomplished by preliminary injections of living cells (embryo skin, blood, etc.) into animals to which the growths were to be transplanted. The animals were thus rendered more or less refractory, like hosts in which the tumors had previously retrogressed. Acting upon this knowledge, considerable quantities of rabbit embryo skin were repeatedly injected into one group of rabbits; the Brown-Pearce epithelioma was transplanted to another group to obtain individuals in which spontaneous retrogression of this tumor had taken place; and a third, comparable group to which nothing was done were retained as controls. All were finally inoculated with the papilloma virus, and the resulting growths were observed and their outlines charted from time to time.

Experiment 5.—7 adult gray-brown rabbits of 2000 to 2500 gm. were injected with minced rabbit embryo skin. Three courses of injection were carried out at intervals of 4 to 5 weeks, the last injections being given 2 weeks before the experiment was begun. Each course consisted of 4 subcutaneous injections of 1 or 2 cc. of a heavy suspension of freshly procured and hashed 23 to 26 day rabbit embryo skin. A second group of 7 similar rabbits were kept untreated but under the same conditions, and when the experiment was done 7 more rabbits of the same breed and from the same dealer were utilized, in which good sized Brown-Pearce tumors in the skin, subcutaneous tissues, and muscles had retrogressed 2 to 3 months previously.

The 21 rabbits were inoculated identically by tattooing active 5 per cent virus (Texas strain) into four spots about 2 mm. across on each side. After 15 to 25 days small papillomas appeared at all of the inoculation sites. The developing

growths were charted at intervals of 5 to 7 days until the 11th week after inoculation, when the experiment was discontinued.

There was no discernible difference in the behavior of the growths in any of the 3 groups, either as regards incidence, time of incubation, size, rate of enlargement, or onset or incidence of retrogression. In about half the animals in each group the papillomas grew progressively; in the remainder they did poorly, either retrogressing abruptly after 6 to 7 weeks or dwindling more slowly after 7 to 11 weeks.

The negative outcome of this experiment was not unexpected, since the growths induced by the virus consisted of the animal's own cells. Similar attempts by others to alter the course of established transplanted growths, or of autochthonous tumors arising spontaneously, or as a result of tarring have also failed (6). This fact, and the experiments just reported, illustrate the limitations of artificial immunization with unaltered homologous tissues. These elicit at best a relatively feeble immunological response, one that can hardly be expected to prevail against "spontaneous" tumors or established transplanted growths, nor even against the papilloma, a growth of exceptional vigor, which can be produced by inoculation of the virus in all normal domestic rabbits, irrespective of whether they will constitute favorable hosts later on.

The Influence of Local Conditions

Local conditions often play a part in determining the fate of the papilloma, and they not infrequently influence the phenomenon of retrogression. Small, discrete growths retrogress much more readily than do large, crowded, confluent papillomatous masses on the same animal, a fact which can be best perceived when the virus is inoculated into many individuals by methods which yield large and small growths respectively.

On many occasions Shope virus has been rubbed into broad areas (about 10 x 12 cm.) of scarified skin on the abdomens of normal gray-brown domestic rabbits with result that large, confluent papillomatous growths occupying most of the inoculated areas appeared after 8 to 16 days. When an active virus material had been employed the confluent growths of this sort generally enlarged into enormous masses, and they did not often retrogress. When this did happen, the process began only after months, and then the tumors often dwindled so slowly that more months were required before they disappeared altogether. It was repeatedly noted that they dried down and flaked away slowly from the periphery inwards;

and often an area of papillomatosis which had become confluent secondarily by the apposition of masses growing from separate foci was broken up by the process of retrogression into its component parts, each of the latter then dwindling from its edges toward its center.

When the same virus fluids were inoculated with the tattoo machine at many widely separated points (about 2 mm. in diameter) or rubbed into several small, scarified rectangles (about 3 x 4 cm. in dimension) on the abdomens and flanks of comparable hosts, the later course of events was very different. The growths first appeared as small, rounded papillomas at the tattooed sites, or confluent or semiconfluent, small growths on the scarified rectangles. These all enlarged rapidly. Often in a third or more of the inoculated animals, 3 to 6 weeks after the virus inoculations, the papillomas, then up to 8 mm. in height, abruptly ceased to enlarge and began to dry and dwindle away, and within a few days all had disappeared. In contrast with the group already described, retrogression began soon and was swiftly concluded.

These observations make it plain that retrogression of the rabbit papilloma, like that of growths consequent on implantation (7), takes place predominantly from the periphery inwards; while in confluent growths where large numbers of primarily affected cells are crowded closely together, retrogression is in some way forestalled or hindered. The slow rate at which large growths retrogress has some connection perhaps with the relatively small ratio of periphery to total size.

So pronounced is the response of the papilloma to such local growth-promoting influences as partial excision, local inflammation due to bacteria, or injections of Scharlach R in olive oil (2), as to suggest the possibility that intercurrent local factors may cause the papilloma to grow progressively at some situations, while at others lacking their influence, it would retrogress. To test this possibility multiple, discrete papillomas were produced on the sides of rabbits, and those on one side were stimulated repeatedly while their fellows on the opposite side served as controls.

Experiments 6, 7, and 8.—Three experiments were done, with 14 normal, adult, gray-brown rabbits. Virus was tattooed into the skin at 6 or 8 small spots on each side. 4 to 5 weeks later, when the papillomas had reached a diameter of 3 to 8 mm. and were up to 5 mm. high, those on the left side of each animal were injected with 0.1 cc. of a saturated solution of Scharlach R in olive oil. The dye was put into the base of the growths through a fine, hypodermic needle inserted

at one edge. The injections were repeated at weekly intervals until 5 to 9 had been given.

In 6 of the 14 animals the control papillomas enlarged progressively, as did the injected ones, the latter far more swiftly. The course of events in one of these animals illustrates clearly the extraordinary growth-promoting influence of the Scharlach R injections. Figs. 3 and 4 show the growths of this animal on the 183rd day after virus injection. 8 injections of 0.1 cc. of Scharlach R in olive oil had been made into the papillomas on the left side between the 26th and 75th days, those on the right being left undisturbed. On the 61st day one growth on each side was removed from corresponding situations for histological study, but these later recurred. When the photographs were taken the undisturbed growths were ordinary, small, discrete papillomas consisting mostly of keratinized peaks deeply cleft and dry almost to their bases; while the injected growths had become

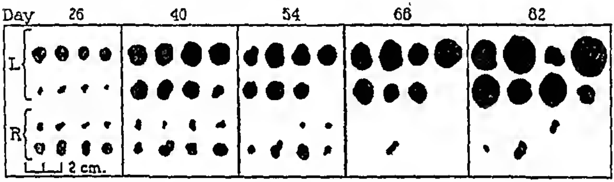

CHART 6. The early course of the growths of D.R. 21 (Experiment 8). The papillomas on the left side (L) were repeatedly injected with Scharlach R in olive oil; those on the right (R) were left undisturbed. For the later course of events see Figs. 5, 6, and 7.

huge, coalescent, fleshy discs, studded with innumerable "pearls." Their surfaces were ulcerating and weeping, and practically devoid of keratinized material, with large, rounded, subepidermal protrusions extending laterally from their bases in many places.

In 7 of the 14 animals the injected and control growths retrogressed simultaneously 4 to 6 weeks after the virus inoculations, but in two instances they disappeared rather slowly and the papillomas that had been injected, and in consequence had reached a larger size, required 7 to 14 days longer to disappear completely than did the control growths on the same animals.

Striking differences in the course of the injected and control growths were observed in the remaining rabbit (D.R. 21, Figs. 5, 6, and 7). In this animal vigorous, discrete papillomas had appeared at all of the inoculated sites. Those on the left side were injected with 0.1 cc. of a saturated solution of Scharlach R in

olive oil on the 26th, 33rd, 40th, 47th, 54th, and 61st days after the virus inoculations, whereas those on the right were not interfered with. Their course during the first 82 days is shown in Chart 6. It will be seen that the control papillomas on the right side of the animal became smaller and disappeared between the 40th and 68th days, leaving flat, smooth, pigmented scars,—except for one, which remained as a small, dry, scab-like wart. During this period the injected growths stopped enlarging, some dwindled slightly, and the smallest became obscured by the reactive proliferation called forth by the Scharlach R injections. Between the 68th and 82nd days this growth reappeared, however, and later it grew to a considerable size. While it was doing so, growths reappeared on the right side at two situations, and the one already present enlarged a little. Photographs (Figs. 5 and 6) taken on the 91st day show the stimulated growths as 8 large, fungoid papillomatous masses, and those on the control side as 2 small, scab-like warts (w) with 3 flat, pigmented scars (s) marking the situations of papillomas that had disappeared. By the 121st day the surface growths on the injected side were dwindling rapidly; underneath them at this time, however, small, rounded, smooth, subcutaneous nodules (pearls) had appeared, and a biopsy of one of them on this day revealed an epidermal cyst 0.8 cm. in diameter, filled with lamellated, keratinized scales and surrounded by a somewhat irregular rind of differentiating papilloma tissue up to ½ mm. thick, embedded in dense reactive tissue consisting largely of fibroblasts. 3 weeks later the surface growths on the injected side had disappeared entirely, as had also the small, dry scabs from the control side. At this time subcutaneous cysts, 0.2 to 2.0 cm. in diameter, were to be seen at 6 sites on the left side, pushing up the scars that marked sites whence the stimulated surface papillomas had retrogressed. These cysts persisted for many months, often rupturing spontaneously through the skin to discharge yellowish, dry, lamellated material. 2 of them received 4 injections of 0.1 cc. of Scharlach R in olive oil during the 8th month, but they were not notably changed thereby. Fig. 7, taken on the 280th day, shows the cysts then present at 5 sites. The rabbit was killed 13 months after the virus inoculation and 9 months after the last of the surface growths had disappeared. Although no stimulating injections of Scharlach R had been given for 4 months prior to the animal's death, several of the cysts persisted. At autopsy, 3 of them were present 1 to 3 cm. in diameter, and one had ruptured through the skin and was discharging lamellated material. The microscope showed that all were simple cysts filled with keratinized scales and lined with a living, proliferating layer of papillomatous epithelium encapsulated in sclerotic connective tissue.

Retrogression occurred in 6 rabbits in these experiments. In 3 it involved the papillomas injected with Scharlach R and the control growths simultaneously, whereas in 2 others the injected papillomas persisted for a few days after the control growths had disappeared

completely. In the sixth instance, however, the injections caused the papilloma cells to proliferate for many months after the control growths on the same animal had retrogressed entirely. In this case the resistance developed by the host against the papillomas was of but moderate degree, and under these circumstances the Scharlach R injections proved the determining element in their fate.

It is plain that favorable local conditions can influence notably and even decisively the fate of the papilloma. Occasionally they may account for the persistence of growths which might otherwise be overcome by a retrogressive influence of generalized character. Such exceptional instances appear analogous to those previously cited of transient intercurrent retrogression, in which only the smaller of a number of discrete growths disappeared, the larger persisting, though much diminished in size, and eventually growing again. Evidently in some instances the balance is finely drawn between the proliferative activity of the enlarging papillomas on the one hand, and the resisting host forces on the other. When this is the case, local conditions may prove decisive for the fate of the growths.

DISCUSSION

Virus-induced tumors are the result of a singular partnership, with cells and virus working actively together to produce a disease. When conditions are favorable to both, vigorous growths arise; but such conditions do not always obtain. The experiments here reported show that conditions sometimes become bad for the cells of the rabbit papilloma, although remaining good for the virus associated intimately with them; and in these instances the growths retrogress, despite the sustained pathogenicity of the virus.

Retrogression of the rabbit papilloma is the result of a generalized resistance of host origin, elicited by the proliferating cells (Experiment 4); but this resistance is distinct from that directed against the virus itself, as manifested in the development of humoral, antiviral antibodies. These antibodies neutralize the virus *in vitro*, yet they do not influence the course of the growths it induces. In some rabbits the growths enlarge progressively although nourished by blood of high antiviral potency, whereas in others in which the antiviral titer

of the serum is comparatively low, they dwindle and disappear (3). The living papilloma cells evidently protect the papilloma virus, as other cells do other viruses (4), and apparently they protect it amply. If humoral antibodies ever neutralized the virus associated with papilloma cells not otherwise damaged, one might expect these to revert to the normal; but no evidence of any such occurrence has been encountered in the many sections of retrogressing growths examined with this point in view. The papilloma cells remain such until they die.

In what way can retrogression of the papillomas take place despite the continued presence of highly pathogenic virus in association with them? The histological changes about retrogressing papillomas are identical with those taking place where other proliferating tissues (neoplastic or non-neoplastic) retrogress in consequence of resistance directed against the cells. Although the precise character of the host reaction responsible for retrogression is still uncertain,[2] it is known, in the case of transplanted tissues, to be the expression of resistance elicited by and directed against the proliferating cells (10).

During retrogression some transplanted tumors grow smaller by disappearance of their peripheral tissue, and the multiple growths of any one animal retrogress simultaneously (7). Both these phenomena are to be observed in the case of the papilloma. The host influence responsible for the retrogression of transplanted growths is sometimes transient in its effects, with result in alternate enlargement and dwindling (10), and this is true of the papilloma as well. When host resistance is weak neither transplanted growths nor the virus-induced papillomas need adjuvant influences to enable them to grow, and when it is strong none of the influences thus far tested can prevent their retrogression; but when it is indecisive inter-

[2] Woglom (8) has reported the recovery and partial purification of an agent from the spleens of rats rendered resistant to a transplanted tumor which will act against tumor cells *in vitro*; and the recent experiments of Gorer (9) provide evidence that the resistance effective against transplanted tumor cells, at least in certain instances, is the result of an immunological response elicited because of antigenic differences between the tissues of the animal in which the growth arose, and those of its new host.

current factors influencing for good or ill the proliferation of the growths may determine their fate, as the present work and previous experience have shown (10). From this it follows that retrogression of the papilloma, though affecting the animal's own cells, is similar in its manifestations to the retrogression of transplanted tumors; and good reason exists for supposing that it is brought about by the same means, namely by a host resistance directed against the cells.

Lacassagne has recently found that retrogression of the rabbit papilloma can be brought about with Roentgen rays in dosages which suffice to kill the papilloma cells *in situ*, but do not harm the virus *in vitro* (11). The retrogression due to the rays, like that which is the outcome of natural forces, as described in the present paper, must be referred to conditions that are bad for the cells but ineffective against the virus associated with them. Woglom has pointed out (10) that during retrogression of transplanted tumors cell division often still goes on in some parts of the growth, as if they were still in good state. It is doubtless in such regions, which have also been noted in retrogressing papillomas, that the virus persists in active form.

Individuals do not ordinarily manifest resistance directed against their own tissues, but this phenomenon is not unknown. Certain viruses cause alterations in the constituents of cells which they infect, with result that these become autogenous antigens and elicit immunological responses (12); and resistance manifested by an individual against its own tissues has been encountered in other states (autohemagglutination, paroxysmal hemoglobinuria), either as a natural occurrence or induced experimentally (13).

Factors similar to those influencing the course of the rabbit papilloma can be discerned in the case of the virus-induced tumors of fowls. The vigor of these growths varies both with the pathogenicity of the virus inoculum, and with the susceptibility of the cellular fabric upon which it acts (young and old hosts, different breeds). In some fowls conditions are favorable both to the virus and to the cells that become infected with it, and in these individuals the growths are largest. In others, less fortunate conditions obtain and retro-

gressing growths are got, the histological changes then indicating that the retrogressive influence is directed against the cells. The cells of retrogressing fowl tumors do not revert to the normal, but remain such until they die, just as happens in the case of the papilloma. The fowl tumor cells protect the causative viruses from the action of humoral antiviral principles, as do the papilloma cells (14). These various phenomena illustrating the respective rôles of virus, cells, and host provide a close parallel with the state of affairs in the virus-induced papillomas of rabbits. It seems probable that the same general principles will be found to hold good in the case of other tumors due to viruses.

SUMMARY

An experimental analysis of the factors responsible for the observed differences in the course of virus-induced papillomas of the rabbit has shown that some are referable to the virus, others to the cells, and yet others to host influences. The interplay of these factors affords enlightening illustration of the nature of the cell-virus relationship in virus-induced tumors. Retrogression of the rabbit papillomas appears to be consequent on a generalized resistance of host origin, elicited by and directed against the proliferating, virus-infected cells.

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EXPLANATION OF PLATES

Plate 21

Figs. 1 and 2. To illustrate individual differences in the epidermal response to proliferative influences. The specimens were procured from 2 rabbits 5 days after the last of 3 intradermal injections of Scharlach R into a skin site previously normal. Such repeated injections had been made at 8 spots in each animal, and the specimens photographed are representative. One of the rabbits carried large and vigorous virus-induced papillomas, whereas in the other, inoculated at the same time and with the same material, the growths had retrogressed after brief proliferation. It will be seen that the epidermis from the individual with vigorous papillomas (Fig. 1) exhibits far the greater hyperplasia. ×8.

Figs. 3 and 4. To show the effect of injections of Scharlach R in olive oil on enlarging papillomas. Fig. 3 shows the ordinary, discrete papillomas existing on one side of a rabbit 183 days after virus had been tattooed into the skin at 8 spots. × ¾. Fig. 4 shows growths of the same origin on the opposite side of the animal, which had received 8 injections each of Scharlach R in olive oil at weekly intervals between the 26th and 75th days. The undisturbed growths are mere, small papillomas, consisting mostly of keratinized cleft peaks, dry almost to their bases, while the injected growths have become huge, coalescent, fleshy discs with ulcerating surfaces and many nodular, subepidermal extensions from their bases. × ¾.

1

2

3

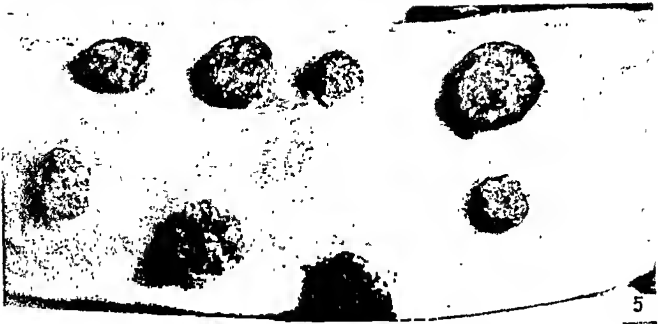
4

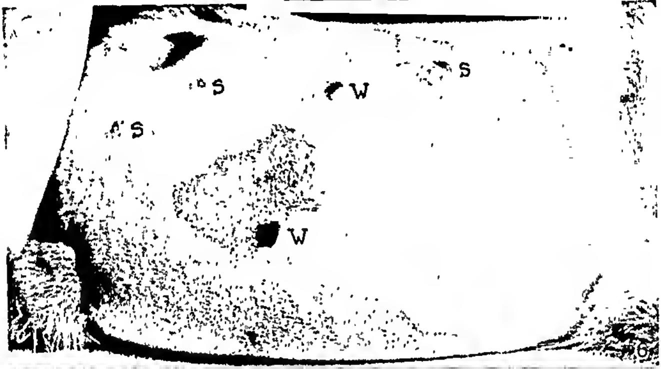
Photographed by Joseph B. Haulenbeek and Louis Schmidt

(Kidd: Course of virus induced rabbit papillomas)

Plate 22

Figs. 5, 6, and 7. To show the influence of injections of Scharlach R in olive oil on the papillomas of a rabbit with moderate resistance to the growths. Fig. 5 shows growths which had become large, fungoid, papillomatous discs in consequence of injections of the dye (D. R. 21—91st day). × 3/5. Fig. 6 shows the uninjected growths on the opposite side of the animal on the same day. 2 small, dry scab-like warts (w) alone remain of the 8 original growths. The sites of 3 others are marked by flat, pigmented scars (s). × 3/5. The scurfy areas near the centers of Figs. 5 and 6 mark the sites where the Scharlach R preparation had been injected into normal skin. Fig. 7 shows subepidermal, papillomatous cysts persisting at several situations where the superficial papillomas of Fig. 5 had long since retrogressed, as had the growths on the opposite side. 280th day. × 3/5.





Photographed by Joseph B. Haulenbeek

(Kidd: Course of virus-induced rabbit papillomas)

A STUDY OF THE MACROPHAGE REACTION IN THE PULMONARY LESIONS OF DOGS WITH EXPERIMENTAL PNEUMOCOCCUS LOBAR PNEUMONIA

By O. H. ROBERTSON, M.D., and CLAYTON G. LOOSLI, M.D.

(From the Department of Medicine and the Douglas Smith Foundation for Medical Research of The University of Chicago, Chicago)

PLATE 23

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At the time of recovery from experimental canine lobar pneumonia there occurs a characteristic change in the fixed tissue cells of the infected lung which we have designated as the macrophage reaction. Certain aspects of this cellular transformation have led to the supposition that it is intimately associated with the mechanism of recovery; namely, the regularity of its occurrence, the progressive diminution in numbers of pneumococci accompanying its evolution and the constant ensuing resolution of the pneumonic lesion (1, 2). However, the cellular change is not confined to the lung lesions of animals recovering from the experimental disease. Just as in the study of the postmortem lungs of patients dying of lobar pneumonia (3) we have frequently found a well developed macrophage response in the pneumonic lesions of dogs which succumbed to the infection. Does this mean that the process of recovery may be initiated in fatal cases but does not become sufficiently widespread to terminate the infection, or are there other unknown factors which play the decisive rôle in bringing about recovery?

In an attempt to gain more insight into the significance of the macrophage reaction a histological study has been made of the pulmonary lesions of 65 dogs dying spontaneously or killed at various stages of the experimental disease.

Materials and Methods

The details of the technique of producing experimental lobar pneumonia in the dog have been described previously (4). The dosage varied from 0.001 cc. to 1.0

cc. of culture of pneumococcus Type I. In most instances x-rays, white blood counts and blood cultures were made daily until death. Autopsies were performed as quickly as possible after death, in many instances, immediately after the animal had expired. The postmortem examination included the heart and abdominal organs and in those cases showing a bacteremia without obvious complications the brain case was opened. Numerous large sections were taken from the various lobes involved and were fixed for the most part in 5 per cent acetic acid-Zenker solution but also in Zenker-formol for a period of 10 hours. They were stained with the Wallace modification of Gram-Weigert's stain (5), and also with hematoxylin-eosin-azure II. In a number of instances including all those in which whole lobes were sectioned, the vessels of the lungs were filled by Loosli's method (6) of clamping the aorta and running fixing fluid, usually Zenker-formol, immediately down the trachea. In this manner the capillaries are normally distended by the blood cells and the free exudate cells fixed before they have had time to round up. Practically instantaneous death with a minimum of circulatory disturbance was produced by the rapid intravenous injection of 500 to 600 mg. of nembutal.

For the purposes of comparison the varying degrees of the macrophage reaction were divided roughly into three categories and designated in terms of + to +++. A beginning reaction, termed +, was characterized by an irregular thickening of the alveolar septa with the great majority of the macrophages still attached to the walls. In a moderately advanced reaction, ++, many of the macrophages were free in the exudate but the polymorphonuclear leucocytes were still the predominant cell type. In a +++ reaction the intra-alveolar exudate was principally macrophagic in composition and accompanied by clearing of the air spaces. These several stages are shown in Figs. 1 to 3. A fourth category, ±, has been employed to indicate the slightest detectable evidence of a beginning reaction. This consists in the presence of occasional alveolar septa containing more large mononuclear cells than are normally observed.

It should be pointed out that the present study deals primarily with changes in the fixed tissue cells of the pulmonary tissues leading to the liberation of alveolar macrophages. Evidence that this is not the only source of macrophages occurring in the pneumonic exudate will be presented in a forthcoming communication by one of the authors.

The Macrophage Reaction in Animals Sacrificed during or Immediately after Recovery

Six dogs were sacrificed during or shortly after recovery (Table I). Two of

these, 90N and 50B, were killed before the recovery process was completed. Their temperatures had reached normal but the pulse rates were still elevated. Dog 90N had an unusually brief disease, recovery beginning at 36 hours. A beginning to moderately advanced macrophage reaction (Figs. 1 and 2) was found to be present in parts of each of the two lobes involved. In the older lesion, right lower lobe, very few pneumococci could be detected and these were all intracellular and undergoing digestion principally within the macrophages. In the younger metastatic lesion of the right middle lobe, microorganisms were present more frequently but chiefly within the phagocyting cells. However, there were some areas of edema in this lesion which showed great numbers of free pneumococci, indicating that the infection was not yet completely terminated. Pneumococci were uniformly found to be more numerous in areas showing a polymorphonuclear cellular exudate than in regions where the macrophages were being mobilized.

The process of healing had apparently reached a more advanced stage in dog 50B killed at 48 hours, since pneumococci were rarely detected in the lesions and these were seen to be in the phagocyting cells. Cultures of the lesions were sterile. This animal showed a more generalized macrophage reaction than did 90N.

One animal, 73R, was sacrificed approximately at the time clinical recovery was complete on the 77th hour after infection when the temperature and pulse had reached normal. Both the involved lobes showed a fairly generalized macrophage reaction, beginning to moderately advanced in degree. In the areas showing macrophages, intact pneumococci were infrequently seen but in the other regions characterized by a polymorphonuclear exudate, microorganisms were present in moderate numbers and all intracellular. Cultures of the lesions yielded pneumococci from the right lower (initial lesion) but none from the right middle lobe.

Two dogs, Nos. 94Q and 35B, were killed within a few hours after recovery. As shown on the table, only a rare intracellular pneumococcus was detectable in the lesions of 94Q and none were seen in those of 35B. However, cultures of the latter lesions yielded a growth of pneumococci. On the other hand, cultures of the lung, bronchi and trachea of dog 94Q were sterile. Both dogs showed a fairly generalized macrophage reaction in the lung lesions.

Dog 91N, having survived an attack of the experimental disease lasting 5 days, was killed 24 hours after recovery. All three diseased lobes, including the right upper which became involved 3 days later than the other two, showed a pronounced macrophage reaction accompanied by beginning resolution (Fig. 3). No pneumococci were found in the lesions either by microscopical examination or by culture.

In summary, dogs killed at the time of recovery from experimental pneumonia all showed the presence of a macrophage reaction in the lung lesions. This was more pronounced in degree in the animals with the longer disease course or in those sacrificed after the termination

TABLE I

Dogs Sacrificed during or Immediately after Recovery

Dog No.	Infecting dose of culture*	Length of disease course	Time of death	Lobes involved and state of lesion	Presence and stage of macrophage reaction	Presence and state of pneumococci in: Areas showing macrophages	Presence and state of pneumococci in: Areas predominantly polymorphonuclear	Additional data
	cc.							
90N	0.05	36 hrs.	36 hrs. during recovery†	R.L. = complete, beginning clearing	R.L. = 0 to ++	R.L. = very few, in cells, with digestion	R.L. = very few, in cells, with digestion	
				R.M. = early lesion	R.M. = 0 to + focal	R.M. = many, in cells, digestion in macrophages	R.M. = many, all in cells, but little digestion	R.M. shows areas of edema with great numbers of free pn.
50B	0.04	48 hrs.	48 hrs. during recovery†	R.L. = complete, beginning clearing	R.L. = + to ++	R.L. = rare, in cells, with digestion	R.L. = rare, in cells, with digestion	R.L. culture = pn.
				R.M. = atelectatic, uneven	R.M. = +	R.M. = as R.L.	R.M. = as R.L.	
94Q	0.001	Less than 48 hrs.	48 hrs. just after recovery	R.L. = complete, beginning clearing	R.L. = +	R.L. = rare, in cells, with digestion	R.L. = rare, in cells, with digestion	Cultures of R.L. trachea and bronchi = 0 M.P. at death = 0
35B	0.02	48 hrs.	54 hrs.	R.L. = complete, beginning clearing	R.L. = ++	R.L. = none	R.M. = none	R.L. culture = pn. R.M. " = pn. Smears from R.L. bronchus = pn.
				R.M. = atelectatic, uneven	R.M. = +			

73R	0.02	77 hrs.	77 hrs. at recovery	R.L. = complete, beginning clearing R.M. = patchy, beginning clearing	R.L. = + to ++ R.M. = +	R.L. = few, in cells, with digestion R.M. = as R.L.	R.L. = few, in cells, with digestion R.M. = as R.L.	R.L. culture = pn. R.M. " = 0 M.P. at death = 0
91N	0.05	5 days	6 days	R.L. = complete, clearing R.M. = complete, 5 days old, clearing R.U. = complete, 3–4 days old, clearing	R.L. = +++ R.M. = ++ R.U. = +++	R.L. R.M. } = none R.U.	R.M. = none	Cultures of all lobes = 0 M.P. 5 days = 0 M.P. 6 " = 10^{-4}

0 = not present or negative. ± to +++ = grades of macrophage reaction. See page 576.

State of lesions includes observations on both the gross and microscopic appearance of the lobe:

(1) Complete and patchy refer to gross appearance of consolidation.

(2) Even, uneven, sparse are used to describe the microscopic appearance of the intra-alveolar cellular exudate predominantly polymorphonuclear in character until the stage of beginning clearing or resolution. In the lesions designated as uneven many alveoli and groups of alveoli are filled with edema fluid characteristic of the early lesion or the later irregular diffuse pneumonia. Sparse indicates few cells and much edema.

M.P. = mouse protective action of dog's serum. The figures 10^{-4} etc. indicate the dilution of a standard pneumococcus suspension, containing 1 billion pneumococci per cc. against which the serum protects mice.

Pn. = pneumococci. R.M. = right middle lobe. L.L. = left lower lobe. P.C. = post cardiac lobe.
R.L. = right lower lobe. R.U. = right upper lobe. L.U. = left upper lobe.

Numbers of pneumococci. Few = less than 20 per oil immersion field, usually less than 5 to 10.
Many = more than 20 per field.
Great numbers = scores to hundreds per field.

W.B.C. = white blood cells. Polys. = polymorphonuclear leucocytes.
B.C. = blood culture. Col. = colonies of pneumococci per 1 cc. of blood.

* All doses of culture were suspended in 1 cc. of starch unless designated otherwise.

† Temperature had reached normal but pulse was still elevated.

of clinical recovery. Pneumococci were found in relatively small numbers in the lesions. They were more frequent where the polymorphonuclears predominated and less numerous or absent in the areas of macrophage reaction. The macrophages were observed to be actively phagocytic and apparently possessed greater powers of digestion than did the polymorphonuclear leucocytes since in many lesions the only intact pneumococci were found within the latter cells. The lung lesions were sterile within 24 hours after recovery. This finding has been corroborated in other animals not listed in the table.

Lung Findings at Sacrifice during the Course of the Disease

With a view to determining the stage in the disease at which the macrophage reaction first appeared and the conditions of the infection which might influence its occurrence, eight animals were killed at varying periods after the inception of experimental lobar pneumonia while still febrile. In five of these the prognosis was considered good in view of the fact that at the time of death the blood was sterile, the white blood count elevated and the pulmonary lesion of limited extent. In three dogs the prognosis was doubtful or definitely unfavorable as indicated by leucopenia or bacteremia or both.

In the dogs with a good prognosis a beginning reaction of the macrophages was detected as early as 24 hours after the inception of the infection (Table II). Although this was slight and focal in character it was associated with the typical changes in the number and distribution of the pneumococci already described. A dog, No. 13R, sacrificed at 39 hours showed a similar picture. In the lesion of a third animal, 63N, killed at 48 hours, a greater proportion of the pneumococci was intracellular than was the case with the earlier lesions, although the total number of microorganisms appeared to be about the same. The 4 day old lesion of dog 9S showed much more pronounced changes in the cellular exudate. In parts of the initial lesion (right lower lobe) a well developed macrophage reaction was present, associated with beginning resolution. On the other hand, the younger metastatic lesion of the right upper lobe exhibited only a focal beginning macrophage response. Pneumococci were plentiful in this lobe, many of them free in the edema fluid. In the initial lesion they were much less abundant and almost all intracellular. By the 6th day the changes in the cellular and pneumococcal distribution were even more pronounced. The two older lobe lesions (right lower and right middle) of dog 79R showed a marked macrophage reaction and resolution. No pneumococci other than fragmentary forms principally within the macrophages could be detected. Cultures of these lobes were sterile. In the younger lesion of the right

upper lobe the macrophage response was irregularly distributed; however, no pneumococci could be found even in the polymorphonuclear exudate, but cultures yielded pneumococci. It would seem that the activity of the pathological process had practically ceased although the animal was still febrile. It will be noted that this was the only one of the four animals tested whose serum showed even a minimal degree of mouse protective action at the time of death.

The three dogs with a dubious or unfavorable prognosis were sacrificed at 24, 48 and 72 hours respectively. The 24 hour lesion of dog 59N (outlook dubious) showed no evidence of a macrophage response. Pneumococci were abundant in the inflammatory exudate, both intra- and extracellular. However, in dog 14D, killed at 48 hours, there was a beginning focal macrophage reaction. Pneumococci were less abundant than in the lesion of 59N. Dog 65R, the only one of the three with an unquestionably bad prognosis (overwhelming bacteremia) yielded the most unexpected findings in the lungs. All three lobes involved showed a generalized macrophage reaction and the absence of pneumococci except for intracellular fragmenting forms. It would appear that the lung infection in this animal had been brought under control despite the rapidly increasing bacteremia. This finding will be discussed further in connection with those of certain dogs dying late in the course of the disease.

A comparison of the pulmonary lesions secured from dogs exhibiting a favorable prognosis with those of animals in which the outlook was dubious or definitely unfavorable for recovery failed to reveal any important difference between the two groups except in respect to the time at which the macrophage reaction first appeared. The finding of a beginning focal reaction in the 24 hour lesion of the dog probably destined to recover, in contrast to the absence of such a change in the lesion of equal age in a dog with doubtful prognosis may be of significance in relation to ultimate recovery as indicated by the data in Table III. However, the fact that the development of conditions unfavorable to recovery from the infection does not suppress the occurrence of the macrophage reaction at a later period suggests that the time of its appearance may be only one of the factors in determining the outcome.

Mouse Protective Action of Serum

The mouse protective action of the serum was tested in the seven dogs killed before or on the day of recovery. In only one instance,

Dogs Sacrificed during Disease

Dog No.	Infecting dose of culture and site	Length of disease course	Lobes involved and state of lesion	Presence and stage of macrophage reaction	Presence and state of pneumococci in: Areas showing macrophages	Presence and state of pneumococci in: Areas predominantly polymorphonuclear	Additional data
			A. With good prognosis*				
	cc.						
95Q	0.001 R.L.	24 hrs.	R.L. = complete, even R.M. = small patch, even	R.L. = 0 to + focal and slight R.M. = as R.L.	R.L. = few, both free and in cells, with digestion R.M. = as R.L.	R.L. = many, both free and in cells, some digestion R.M. = few, mostly in cells, some digestion	Consecutive sections of whole R.L. show only 2 small areas of beginning macrophage reaction
13R	0.001 R.L.	39 "	R.L. = complete, even	R.L. = 0 to + focal and slight	R.L. = few, both free and in cells, with digestion	R.L. = many, mostly in cells, but little if any digestion	Consecutive sections of whole lobe show macrophage reaction confined to distal ⅓ of lobe M.P. at death = 0
63N	0.05 R.L.	48 "	R.L. = complete, even R.M. = as R.L.	R.L. = 0 to + focal	R.L. = many, but less than poly. areas, all in cells and digestion in macrophages	R.L. = many but mostly in cells, some digestion. Small areas of edema with many free pn.	
9S	0.02 R.L.	4 days	R.L. = complete, beginning clearing R.M., P.C. = complete, even R.U. = complete, uneven	R.L. = + to +++ R.M. = + to ++ R.U. = 0 to +	R.L. = rare, in cells, fragmenting R.M. = as R.L. R.U. = few, mostly in cells	R.L. = few in cells, with some digestion in polys. R.M. = R.L. R.U. = many, free in edema and in polys.	M.P. at death = 0

79R	0.001 R.L.	6 days	R.L. } R.M. } = complete, clearing R.U. = complete, even	R.L. = +++ R.M. = +++ R.U. = 0 to ++	R.L. = only fragments of pn. in macrophages R.M. = as R.L. R.U. = " "	R.U. = very few, in cells, with digestion	R.L. culture = 0 R.M. " = 0 R.U. " = pn. M.P. before infection = 0 M.P. at death = 10^{-8}
B. With dubious or bad prognosis†							
59N	0.05 L.L.	24 hrs.	L.L. = complete, uneven	L.L. = 0		L.L. = many, both free and in cells	W.B.C B.C. Before infect. = 26,000 At 24 hrs. = 5900 0
14D	0.05 R.L.	48 "	R.L. = complete, even R.M. = as R.L.	R.L. } R.M. } = 0 to +	R.L. = few and all in cells, with digestion R.M. = as R.L.	R.L. = many, mostly in cells, little if any digestion R.M. = as R.L.	W.B.C. B.C. Before infect. = 9000 At 24 hrs. = 3500 0 " 48 " = 6000 4 col.
65R	0.001 in 2 cc. starch	77 "	R.L. = complete, beginning clearing R.M. = complete, even R.U. = patchy, uneven	R.L. = + to +++ R.M. = + to ++ R.U. = + to ++	R.L. = only in cells, fragments R.M. = as R.L. R.U. = " "	R.M. = very few in cells, with digestion in macrophages and polys. R.U. = as R.M.	W.B.C. B.C. Before infect. = 17,600 At 24 hrs. = 8600 100 col. " 48 " = 14,600 750 " " 72 " = 14,800 960 "

* Well sustained white blood count and sterile blood.

† Leucopenia or bacteremia or both.

TABLE III

Dogs Dying within 21 to 50 Hours

Total No. of dogs and time of exitus	Infecting dose of culture	Lobes involved	Presence and stage of macrophage reaction	Presence and state of pneumococci in		Blood cultures
				Areas showing macrophages	Areas predominantly polymorphonuclear	
8 21–29 hrs.	0.02 to 0.8 cc. in 6 cc. starch*	4 to 6	0	—	Great numbers, largely free	In most instances more than 1000 col.
7 36 hrs. (about)	0.02 to 0.6 cc. in 6 cc. starch†	4 to 6	0	—	Many to great numbers, both free and in cells	At 24 hrs. = 4 to 1000's col. " autopsy all = +
3 36 hrs. (about)	0.001 to 0.02 cc. in 1 to 6 cc. starch‡	2 to 6	±	Many, chiefly in cells	Many, both free and in cells	At 24 hrs. = 0 to 1 col. " autopsy all = +
9 41–50 hrs.	0.001 to 0.6 cc. in 1 to 6 cc. starch	3 to 6	+ focal	Few to many, chiefly in cells. Active phagocytosis and digestion by macrophages	Many both free and in cells, active phagocytosis by polys. but little digestion	At 24 hrs. = 0 to 1000's col. " 48 " = 200 to 500 col. " autopsy all = +

* The suspension of a given dose in 6 cc. of starch produces a much higher mortality than does the same dose in 1 cc.

† One dog received 1 cc. of culture in 1 cc. of starch.

‡ Two dogs received 1 cc. of starch and one, 6 cc. of starch.

dog 79R, Table II, was this property found to be present and that was of a minimal degree.[1] The serum of the other animal, No. 91N, showed mouse protection on the day following recovery. These findings are in harmony with previous observations, namely, that specific acquired humoral immunity is uncommonly detectable at the time of recovery from experimental pneumococcus pneumonia in the dog.

The Distribution of the Macrophage Reaction

Consecutive sections of a whole lobe were made of the lesions in five dogs killed at periods from 24 hours to 50 hours after the inception of the infection in order to determine the distribution of this cellular reaction.

With the exception of dog 94Q (Table I) killed at 48 hours, just after recovery, the macrophage reaction was found to be present only in certain parts of the lobe. In this animal the reaction was in its beginning stages, largely focal in character but distributed throughout the lesion. It was most marked in the area where the infection was initiated, which was also true in four other dogs. Of the two animals sacrificed at 24 hours, one (95Q, Table II) with a favorable prognosis exhibited two areas of beginning macrophage response, 2 to 3 cm. in diameter. One of these was in the area of inoculation, the other at the base. The other 24 hour dog, with a dubious prognosis (white blood count of 1350 at the hour of death and the whole right lung involved) showed only a small focus of beginning macrophage response at the original site of infection. Pneumococci were present in much greater numbers in this lesion than in that of the 24 hour dog probably destined to recover. In one animal sacrificed at 40 hours during the stage of active infection but with a good prognosis, the macrophage reaction was confined to the distal third of the lobe. The fifth dog autopsied just after death at 50 hours showed a fairly well distributed beginning focal reaction in the distal fourth of the lobe and a number of small isolated areas scattered throughout the basal and posterior parts of the lesion.

It is evident from these findings that the macrophage reaction in its inception is by no means uniformly distributed throughout the pulmonary lesion but is characterized initially by numerous more or less isolated foci which coalesce and spread to form larger areas of the characteristic cellular change. The relationship between age of lesion and

[1] Most of the tests were run to 10^{-8} of the standard suspension, which when plated out yielded 10 to 20 colonies.

appearance of the macrophage reaction is further borne out by the autopsy studies on dogs surviving for a number of days, namely, that the most advanced reactions were found in the older lesions.

Lung Findings in Animals Dying within 24 to 50 Hours

Examination of the lung lesions of eight dogs dying from 21 to 29 hours of a fulminating pneumonia failed to reveal the presence of a macrophage reaction in any of them. The pulmonary process was usually widespread, involving both halves of the lung and sometimes occupied all six lobes of the dog's lung. The lesions were principally of the irregular diffuse type with large areas of edema and often a relatively scanty cellular exudate. Pneumococci were present in large numbers, sometimes growing in masses and were for the most part extracellular. In several instances a moderate degree of phagocytosis was observed.

The first indications of a beginning macrophage reaction were observed in three of the ten dogs dying at about 36 hours. It was very slight and focal in character (Table III). Pneumococci were abundant throughout the inflammatory process, more numerous in the younger lesions than in the older ones. In most of the initial lesions well marked phagocytosis by the polymorphonuclears was observed and sometimes evidence of slight intracellular digestion could be detected. The only conspicuous difference between the dogs showing the incipient macrophage response and those not showing it was the lesser degree of bacteremia in the former animals.

It was not until the survival time was more than 40 hours that a well defined macrophage response was observed. While the reaction was focal and usually quite limited in extent, it was detectable in the initial lesions of all nine animals dying between 41 and 50 hours after the inception of their pneumonia (Table III). Pneumococci were constantly fewer in numbers in the areas of macrophage proliferation where these cells showed active phagocytosis and digestion of the engulfed organisms. On the other hand, while phagocytosis by the polymorphonuclear leucocytes was often active, intracellular digestion was slight in degree or absent. In no instance did the area of macro-

phage reaction comprise more than a very small percentage of the total inflammatory lesion.

Lung Findings in Animals Dying within 3 to 7 Days

By the 3rd day a wider distribution of the macrophage reaction was observed. Of the six animals dying at this time only one, 6M (Table IV) failed to exhibit this cellular change.[2] The detailed findings in two of the dogs are given in Table IV. The most striking changes were shown by dog 87Q in which a bilateral empyema was present. The single lobe involved exhibited a fairly generalized beginning to moderately developed macrophage response and only a few intracellular pneumococci despite the presence of an overwhelming bacteremia. With the exception of dog 6M, whose blood remained sterile, a pronounced bacteremia was present in every instance.

Dogs surviving until the 4th day exhibited not only a more widespread but a more pronounced development of the macrophage reaction than was observed at earlier periods. A single instance of this stage is listed in the table as representative of the findings in the group. While the macrophage response was present in one or more of the involved lobes there were always certain lesions or areas in the lesions where free pneumococci were observable in areas in which the polymorphonuclear leucocytes constituted the predominant cell of the alveolar exudate. As at the 3rd day stage a marked blood invasion was present in all instances at the time of death.

By the 5th day empyema was much more commonly present. Four of the five dogs dying at this time showed a bilateral purulent pleural exudate. The most notable finding in the group of four dogs with empyema is the marked development of the macrophage response in many of the lobe lesions, together with beginning resolution and the diminution or disappearance of pneumococci from these areas despite

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[2] This dog had recovered from a number of previous infections, the last one several months earlier. On the basis of previous observations in dogs with recurrent infections, one would have expected to find a pronounced early macrophage response in this animal. Another dog receiving this very large infecting dose, lethal in 85 per cent of normal dogs, died on the 3rd day and exhibited a focal though slight macrophage reaction in two of the five lobe lesions.

TABLE IV

Dogs Dying from 3rd to 7th day

Total No. of dogs and day of exitus	Individual dogs	Infecting dose of culture and site	Lobes involved and state of lesion	Presence and stage of macrophage reaction	Presence and state of pneumococci in: Areas showing macrophages	Presence and state of pneumococci in: Areas predominantly polymorphonuclear	Blood cultures
6 on 3rd day	87Q	0.02 cc. in 6 cc. starch R.L.	R.L. = complete, even Bilateral empyema	R.L. = + to ++	R.L. = few, in cells, with digestion	R.L. = few, in cells, with digestion	At 24 hrs. = 3 col. " 48 " = 1000's col. " 72 " = 1000's "
	6M	0.6 cc. in 6 cc. starch R.L.	R.L., R.U. = complete, uneven R.M. = complete L.L. = patchy, sparse	R.L. = 0 R.U. = 0 to ± L.L. = 0	—	R.L. = many, mostly in cells with some digestion R.U. = great numbers, mostly free L.L. = many, free	At 24 hrs. = 0 " 48 " = 0 " autopsy = 0
5 on 4th day	56P	0.02 cc. in 1 cc. starch R.L.	R.L., L.L. = complete, uneven	R.L. = + to ++ L.L. = 0	R.L. = none, except fragments in macrophages	L.L. = many, free	At 24 hrs. = 1000's col. " 48 " = 1000's " " autopsy = +
5 on 5th day	29R	0.02 cc. in 1 cc. starch R.L.	R.L., R.M. = complete, even R.U. = patchy, with abscesses, even L.U. = patchy, uneven	R.L. = 0 to + slight and focal R.M. = 0 R.U. = 0 L.U. = 0	R.L. = few, all in cells, active phagocytosis and digestion by macrophages	R.L. = few, mostly in cells, digestion R.M. = many both free and in cells, no digestion R.U. = great numbers, mostly in cells, no digestion L.U. = as R.U.	At 24 hrs. = 0 " 48 " = 0 " 96 " = 20 col. " autopsy = 0

5 on 6th day	28R	0.5 cc. in 1 cc. gelatin Locke's sol. with mucin L.L.	L.L. } L.U. } = complete, uneven R.L. = patchy with abscess, sparse	L.L. = +++ L.U. = 0 to + R.L. = 0	L.L. = many in macrophages, with marked digestion, rare free pn. L.U. = few, and almost all in cells	L.U. = many, both free and in cells, some digestion in polys. R.L. = many free	At 24 hrs. = 40 col. " 48 " = 420 col. " 5 days = 1000's col.
2 on 7th day	24L	0.25 cc. in 1 cc. starch R.L.	R.L. = complete, beginning focal clearing R.M. = complete, 6–7 day old, even R.U. = complete, 4–5 day old, even L.U. = partial, uneven L.L. = patchy, sparse	R.L. = ++ R.M. = 0 to + R.U. = 0 L.U. = 0 L.L. = 0	R.L. = none, except fragments in macrophages and polys.	R.M. = few, but both free and in cells R.U. = many, both free and in cells L.U. = many, almost all in cells L.L. = few, mostly free, in edema fluid Some digestion in polys. in all lobes	At 24 hrs. = 0 " 48 " = 0 " 96 " = 0 " 5 days = 0 " autopsy = 0
	58S	0.02 cc. in 1 cc. starch L.L.	L.L. } L.U. } = complete, atelectatic clearing Bilateral empyema	L.L. } L.U. } = +++	L.L. = rare fragment in macrophages L.U. = none	— —	At 24 hrs. = 40 col. " 48 " = 800 col. " 72 " = 200 " " 5 days = 1000's col. " 6 " = 1000's " " autopsy = +

the presence of bacteremia. Another unexpected finding was that in the single dog, 29R, (Table IV), without complications and a sterile blood at autopsy, the macrophage reaction was slight, focal and seen only in the initial lesion. However, pneumococci were present in much greater numbers in the lesions of this animal than they were in those of the dogs dying with empyema and bacteremia. This animal was found to have a number of small abscesses in the right upper lobe.

Of the five dogs dying on the 6th day, three showed bilateral empyema. The findings in these animals were essentially the same as in the dogs dying on the 5th day. They all showed a moderately to well developed macrophage reaction in some of the lobe lesions. An example of this stage is shown in the table, No. 28R. All five of the dogs had an extreme degree of bacteremia at death.

Of the two dogs dying on the 7th day of the disease one, 24L, showed no complications or bacteremia but had involvement of practically all its lung tissue. A macrophage reaction was present in the two oldest lobe lesions of this animal (Table IV), the right lower and right middle (the latter was involved by the end of the first 24 hours). No pneumococci except intracellular fragments were seen in the right lower lobe which exhibited a generalized and moderately developed macrophage response. In the right middle lobe intact pneumococci were observed only in those regions in which macrophages were not present. In the three other lobe lesions no macrophage reaction was observed, even in the right upper lobe which had been involved for 4 to 5 days. The lobes of the left lung became diseased within the last 24 hours of the infection. In these latter three lesions, many pneumococci were present both intra- and extracellular. On the other hand, dog 58S dying with a bilateral empyema and overwhelming bacteremia showed a marked macrophage reaction throughout both the involved lobes which were in a state of advanced resolution. Pneumococci had practically disappeared from the lesions.

The autopsy findings on the 50 dogs listed in Tables III and IV can be briefly summarized as follows:

The pulmonary lesions of dogs dying in less than 30 hours were characterized by a diffuse irregular inflammatory process consisting of edema and polymorphonuclear

infiltration with great numbers of pneumococci, mostly extracellular. No evidence of a macrophage reaction was detected in lesions younger than about 36 hours. At this stage there appeared in some of the animals a very slight focal beginning macrophage response. After 40 hours this reaction was observed to be present in some part of the lung lesions in all but one of the 32 animals studied. With few exceptions the reaction became more pronounced and widespread with the increasing age of the lesion. After the 4th day many of the lobe lesions showed a well developed macrophage response with resolution and diminution in numbers or apparent complete disappearance of pneumococci. Such a reaction occurred in the presence of marked bacteremia or empyema or both. In some of these animals all the lung lesions appeared to be completely freed of microorganisms except for intracellular fragments. The presence of bacteremia of course makes it impossible to determine whether or not viable pneumococci are still present in such lungs. The three animals in which the blood remained sterile showed much less response of the macrophages, but many more pneumococci were present in the lesions than was the case of dogs with clearing lungs in the presence of bacteremia.

DISCUSSION

In view of the fact that the macrophage reaction occurs in fatal as well as non-fatal infections what significance can we attach to this response of the lung tissue in experimental lobar pneumonia? The one constant finding throughout the whole study was the associated diminution or disappearance of pneumococci with the increasing concentration of macrophages in the lesion. This would indicate that the lung possesses great innate powers of recovery. Given a sufficient length of life the lung seems to be able to free itself largely or even completely of the invading pneumococci despite their persistence in the blood stream. A second equally clear inference from this study is that the macrophage reaction constitutes only a part of the mechanism of recovery. Control of the local pulmonary infection does not necessarily lead to recovery since the animal may die of a generalized infection with or without complications. On the other hand failure of the macrophage reaction to occur results in the persistence of large numbers of pneumococci in the pulmonary lesions and eventual death even though the infection remains localized in the lungs, a condition observed in three of the 23 animals surviving 3 or more days. Infections which eventuated in recovery were always characterized by the absence of or a transient, limited bacteremia and the occurrence of a macrophage reaction in the pneumonic lesion.

These data suggest that recovery depends on a dual mechanism. First, a generalized process which acts to localize the infection and prevent or control invasion of the blood stream and, second, a local one by which the lesion is finally freed from the invading microorganisms. That the general process may be adequate to deal effectively with certain infections if they can be brought under control early seems evident from the histological and bacteriological findings in the lesions of dogs with a very brief disease course. In such instances the macrophage reaction is usually in its beginning stages and often focal, yet the pneumococci have disappeared.

Concerning the nature of these two aspects of the recovery mechanism our information is by no means complete. It may be presumed that the generalized process consists in part, at least, of the pneumococcidal power of the blood, since animals exhibiting this property to a high degree are the most resistant to pneumococcus infection and disappearance of the pneumococcidal-promoting properties of the serum during the course of experimental pneumococcus infection is accompanied by bacteremia (7). However, certain observations both in experimental animals and human beings suggest that the control of bacteremia may depend on the operation of other factors as well. It was found by Terrell (7) and more recently corroborated by ourselves that in the occasional dog with experimental pneumonia, bacteremia occurs despite the persistence of pneumococcidal-promoting properties of the blood serum. This may mean that under such conditions there is a marked depression of the antipneumococcal activity of the leucocytes and other phagocyting cells of the body. Until further data are available this possibility cannot be excluded. It seems unlikely that there is a lack of union between antigen and antibody, since serum from dogs exhibiting this state when mixed with normal leucocytes *in vitro* is capable of causing the destruction of large numbers of the same strain of pneumococci. The studies of Sutliff and Rhoades (8) on the pneumococcus-killing power of the blood of patients showing bacteremia during the course of lobar pneumonia contribute important information on this subject. They found that the whole blood containing a moderate number of pneumococci often sterilized itself after agitation in the incubator, which suggests that the time element may

be of importance. They found, furthermore, that such defibrinated blood was capable of killing large numbers of added homologous pneumococci. These observations do not seem to be explainable on the basis of depressed activity of the circulating leucocytes.[3]

Whether the findings in human beings are entirely comparable to those in dogs with the experimental disease is not clear. In all the animals and human beings studied thus far, disappearance of the pneumococcidal-promoting properties of the serum has been accompanied by the appearance of pneumococci in the blood. On the other hand we have not infrequently found in patients an absence both of natural antipneumococcal immune substances and bacteremia early in the course of the disease and persisting until the time of recovery, a condition not observed in the experimental animal. These findings serve to indicate the complexity of the mechanism which controls the localization of the pneumococci in the pulmonary lesion.

Likewise, the means by which the local process—macrophage reaction—brings about the destruction of the invading microorganisms has not been completely elucidated. Our observations indicate that the fully developed free macrophages are capable of engulfing and digesting large numbers of pneumococci, but whether or not this action takes place under the influence of newly produced immune bodies we do not know. Suggesting the possibility of some other action of the macrophages is the not infrequent histological finding of diminishing numbers of pneumococci in areas of beginning macrophage reaction when these cells are still largely attached to the alveolar walls and are not detectably phagocytic.

The question of the rôle played by the known acquired antipneumococcal immune substances in the mechanism of recovery has not been included in this discussion which deals primarily with the experimental disease, because these substances are found so seldom in the recovering dog. When immune bodies do appear their occurrence is subsequent to the development of the pulmonary macrophage reaction, as illus-

[3] Several recent observations in this laboratory indicate that the leucocytes of dogs with marked bacteremia are as active in the phagocytosis and digestion of pneumococci as are normal dog leucocytes.

trated by dog 91N (Table I). This suggests the possibility of an excess local production in sufficient concentration to be detectable in the circulating blood.

SUMMARY

A study has been made of the occurrence of the macrophage reaction in the pulmonary lesions of dogs sacrificed during the course of experimentally induced pneumococcus lobar pneumonia or dying as a result of the infection. This characteristic transformation of the fixed tissue cells of the lung was found as a constant accompaniment of recovery. It was also present in varying degrees in the great majority of fatal instances provided the animal lived more than 40 hours. In general the longer the animal survived, the more pronounced the macrophage reaction observed in the lung lesions at autopsy. The numbers of pneumococci in the lesions diminished progressively with the evolution of the cellular change which terminated in resolution of the pneumonic exudate. Some dogs surviving for 4 days or more showed practically complete clearing of the pulmonary lesions but succumbed with an overwhelming bacteremia or empyema or both. On the other hand, several animals dying with a sterile blood, exhibited lesions characterized by little or no macrophage response and the presence of many pneumococci. These findings suggest that recovery from experimental lobar pneumonia in the dog depends on a dual mechanism consisting of a generalized process which prevents or controls invasion of the blood stream and a local one by which the lesion is finally freed from the invading microorganisms. The nature of these two processes is discussed.

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EXPLANATION OF PLATE 23

The tissues were fixed immediately after the animals were sacrificed. The aorta was clamped and the lungs filled with Zenker-formol solution injected by way of the trachea. Specimens were embedded in collodion, sectioned and stained by Maximow's hematoxylin-eosin-azure II method. The relative sparsity of the cellular exudate is to be accounted for by the method of fixation.

FIG. 1. Dog 65R (Table II) killed at 65 hours after the inception of the disease. The area of the right upper lobe lesion shows a beginning macrophage reaction, designated as +, irregular thickening of the alveolar walls due to the presence of large mononuclear cells, certain of which protrude into the air spaces. The cellular exudate is composed almost entirely of polymorphonuclear leucocytes. × 750.

FIG. 2. Dog 65R. Another area of the right upper lobe lesion which shows a moderately advanced macrophage reaction, termed ++. Many of the large mononuclear septal cells have left the alveolar walls and are present in the exudate as typical macrophages. Other smaller mononuclear cells are also seen in the alveolar spaces. × 750.

FIG. 3. Dog 91N (Table I) killed 24 hours after recovery. Right lower lobe lesion shows a pronounced macrophage reaction, +++, which is characterized by the predominance of large macrophages in the exudate. Several are seen to be still attached to the alveolar wall. × 750.

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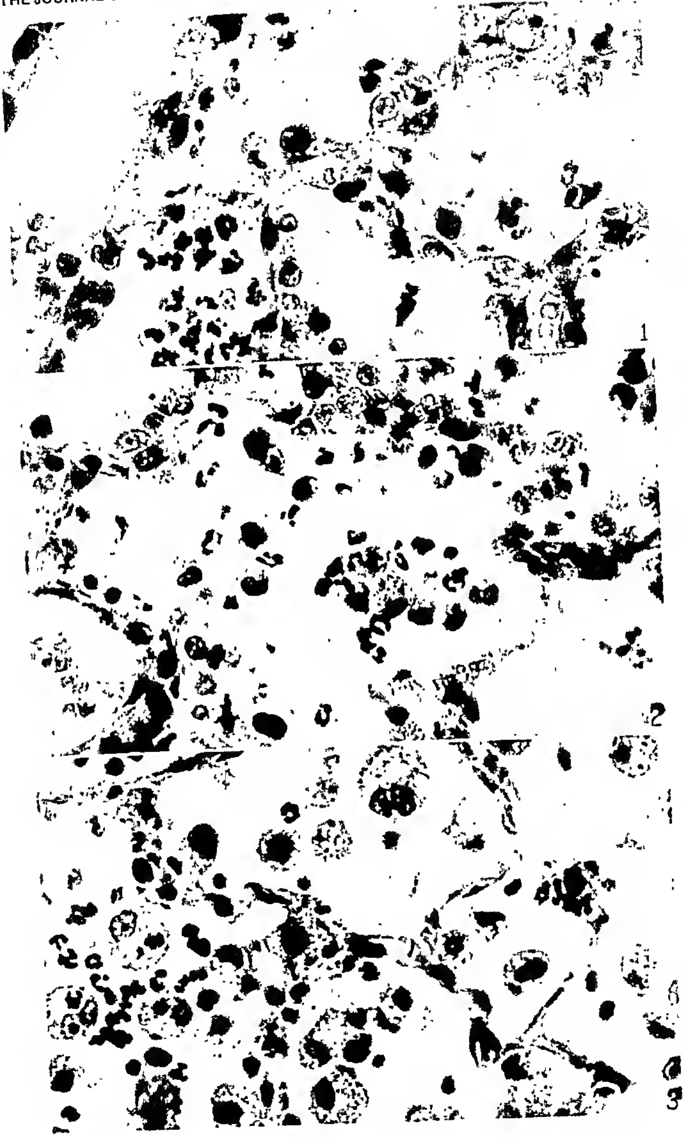
PLATE 23



(Robertson and Loosli: Macrophage reaction in pneumococcus pneumonia)

LOCAL RECOVERY IN EXPERIMENTAL PNEUMOCOCCUS LOBAR PNEUMONIA IN THE DOG

By O. H. Robertson, M.D., and L. T. Coggeshall, M.D.

(From the Department of Medicine and the Douglas Smith Foundation for Medical Research of The University of Chicago, Chicago)

Plates 24 and 25

(Received for publication, January 12, 1938)

In a previously reported study of the occurrence of the macrophage reaction in the pulmonary lesions of dogs with experimental lobar pneumonia (1) it was found that this characteristic reaction of the fixed tissue cells of the lungs was not confined to any one stage of the disease. While it was present regularly at the time of recovery it was also found in some part of the lesion in the great majority of fatally terminating infections, provided the animals lived longer than 40 hours. With few exceptions the reaction became more pronounced and widespread with the increasing age of the pathological process and was accompanied by a progressive diminution in the number of pneumococci, whereas microorganisms persisted, usually in larger numbers, in those lesions or parts of a lesion where this cellular change failed to occur. In certain dogs dying after 4 or more days the consolidated lobes were found to be in a well advanced stage of resolution with an alveolar exudate consisting almost entirely of macrophages. The absence of visible pneumococci in such lesions suggested that the lung had largely or completely freed itself of the invaders. However the constant presence of a concomitant bacteremia or empyema at the time of death made it impossible to determine by culture whether or not pneumococci were still present in the apparently healing lesion.

Since the above observations we have been able to secure additional data on the occurrence of local recovery during the course of favorably progressing experimental pneumonia which have provided us with

much more definite information as to the importance of the rôle played by the macrophages in the disposal of the invading pneumococci. In order to be certain that the clearing of one part of the lesion as detected by x-ray represented an actual local recovery and not just the beginning of the general process of healing, we have selected for study only those instances in which resolution of one lobe lesion was occurring at the time a metastatic process was developing in another lobe. The determination of the cellular reaction, the distribution and numbers of pneumococci in such lesions together with the general humoral response offers a unique opportunity for the further elucidation of the nature of local recovery of the pneumonic lesion. The present study comprises such observations on six dogs killed during the active stage of the disease. Since the phenomenon of simultaneous clearing and spread of the pathologic process was detected in less than 5 per cent of animals infected with doses of pneumococci from which recovery usually eventuated, the six reported instances represent a selection from well over 100 dogs undergoing the experimental disease.

Materials and Methods

The dogs were infected with the Type I pneumococcus used in previous studies. Four of them received 0.02 cc. of culture in 1 cc. of starch, a dose which results in a mortality of about 7 per cent. The other two were given doses of 0.05 and 0.25 cc. respectively. The latter animal had had two previous attacks of the experimental disease and hence was able to resist a dose which is usually lethal in the normal animal. X-rays, white counts and blood cultures were made daily in most instances. The animals were sacrificed by means of the rapid intravenous injection of 500 to 600 mg. of nembutal. Cultures of the various lobe lesions were made immediately after death, often several cultures from a single lobe. In several instances the lungs were fixed *in situ* after ligature of the aorta in order to fill the pulmonary capillaries (Loosli's method (2)). A number of large sections were taken from each lobe, some of which were fixed in 5 per cent acetic Zenker solution and some in Zenker-formol for 10 hours and stained with Wallace's modification of the Gram-Weigert stain (3), and also with hematoxylin-eosin-azure II. Some of the tissues were embedded in paraffin, others in gelatin.

EXPERIMENTAL

Clinical Course of the Disease.—The experimental disease ran much the same course in all six dogs except for duration. Three animals

were sacrificed at the end of 4 days; two at 5 and one at 6 days respectively. The blood remained sterile, the number of circulating leucocytes was well sustained and spread of the pulmonary lesion was gradual. Temperature charts of two of the dogs, 9H, and 9S, are shown in Text-figs. 1 and 2. In three instances there was a drop in temperature preceding the appearance of the final metastatic lesion as shown in Fig. 1. In the other three the febrile temperature persisted until death. Beginning clearing of the initial lesion as indicated by x-ray was detected at 72 hours after the inception of infection in one dog and at 96 hours in the other five.

As an example of the progressive changes in the x-ray appearance of the lesions, the series of films taken on dog 9H are reproduced in Figs. 1 to 4. This animal was infected on Mar. 12, 1934. At the end of 24 hours consolidation of the entire right lower lobe was present. By 48 hours the lesion had spread to involve the right middle lobe. At 72 hours a spread to the left mid-lung field (at autopsy found to be a small left middle lobe) was evident and there was a probable beginning clearing of the initial lesion. By 96 hours there was no doubt as to the occurrence of resolution in the right lower lobe and the metastatic lesion had extended to involve the upper part of the left lung field. The dog was sacrificed immediately after the last x-ray. A film made of the excised lungs showed another small area of consolidation at the tip of the right upper lobe which had not been detected in the x-ray of the lungs made just before death. In several other instances, the presence of small early lesions not suspected during life was discovered at autopsy. This suggests that the phenomenon of clearing and spread may be of more common occurrence than the x-ray findings would lead one to believe.

Findings at Autopsy.—A summary of the autopsy data is given in Table I. The most striking finding was the difference between the clearing and the early spreading lesions in respect to cellular composition and the content and distribution of pneumococci. The clearing (resolving) lesions were sterile and characterized by a well developed macrophage reaction, whereas the early lesions consisted of alternating areas of edema and polymorphonuclear infiltration containing usually many pneumococci both free and intracellular. This contrast is illustrated by the photomicrographs taken from the initial and the most recent lesions of dog 9H (Figs. 1 and 2). The only pneumococci found in the clearing lesions were intracellular fragments. In two instances (dog 59S and 9S) marked differences within a single lobe lesion were observed. This was most strikingly apparent in dog 59S.

TABLE I

Findings at Time of Death in Dogs Showing Simultaneous Clearing and Spread of the Pulmonary Lesions

Dog No.	Lobes involved	Age of lesion	Stage of lesion	Cultures of lesions	Macrophage reaction	Predominant cell of alveolar exudate	Number and distribution of pneumococci	Culture of heart blood	Mouse protective action of serum
9H	R.L.	96 hrs.	Clearing	0	+++	Macrophage	Only intracellular fragments	0	—
	R.M.	72 "	Beginning focal clearing	0	+ to +++	Macrophages and polys. about equal number	" " "		
	L.M.	48 hrs. ±	Intense consol.	0	0 to + slight and focal	Polymorphonuclear	Very few intracellular, mostly fragments		
	R.U.	? age	Patchy consol.	+	0 to +	"	Very few mostly intracellular		
	L.U.	24 hrs. ±	Early consol.	+	0	"	Many, free and in polys.		
100R	L.L.	92 hrs.	Clearing	0	++ to +++	Macrophage	Only intracellular, mostly fragments	0	0
	R.M.	? age	Patchy consol.	+	0 to +	Polymorphonuclear	Few and all intracellular		
	R.U.	24 hrs. ±	Early consol.	+	Lower part = 0	"	Lower part = many free and in polys.		
					Upper part = 0 to +	"	Upper part = few, in cells		
59S	R.L.	5 days	Clearing	0	+++	Macrophage	Only a few, intracellular fragments	0	0
	P.C.*	? age	"	0	++	"	None		
	R.U.	24 hrs.	Upper part = early lesion	+	0	Polymorphonuclear	Great numbers, mostly free		
			Lower part = beginning clearing	0	+++	Macrophage	None		

9S	R.L.	92 hrs.	Clearing	0 post. part + ant. part	0 to +++	Macrophage	Rare, intracellular in macrophage areas. Fairly frequent hoth intra- and extracellular in poly. areas	0	0
	R.M.	24 to 48 hrs.	Intense consol.	+	0 to ++	Polymorphonuclear	Frequent but all intracellular		
	R.U.	? age	Very early consol.	+	0	"	Many hoth free and intracellular		
57L	R.U.	6 days	Clearing	0	+++	Macrophage	None	0	0
	R.M.	48 hrs. ±	Fairly intense consol.	+	+ to ++	Polymorphonuclear	Very few, intracellular, fragmenting		
	R.U.	24 hrs. ±	Early consol.	+	0 to +	"	Moderately frequent, free and in polys.		
69L†	R.L.	5 days	Clearing	+	+++	Macrophage	Only intracellular, fragments	0	0
	R.M.	4 "	Beginning clearing	+	++ to +++	"	Only intracellular, mostly fragments		
	R.U.	24 to 48 hrs.	Intense consol.	+	+	Polymorphonuclear	Frequent intracellular, mostly fragments		

0 = negative.

+ to +++ = grades of macrophage reaction:

+ = beginning reaction, chiefly thickening of alveolar septa due to presence of large mononuclear cells. Few macrophages free in exudate.

++ = moderately advanced reaction, many macrophages free in exudate but polymorphonuclear leucocytes still the predominant cell of the exudate.

+++ = well developed reaction, cellular exudate chiefly macrophagic in character and beginning clearing of the alveoli.

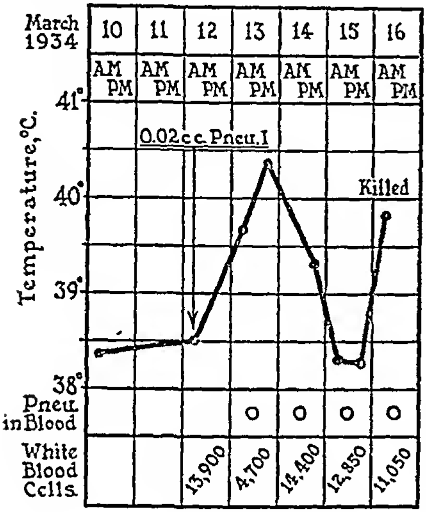
Consol. = consolidation. Polys. = polymorphonuclear leucocytes.

* P.C. = postcardiac lobe.

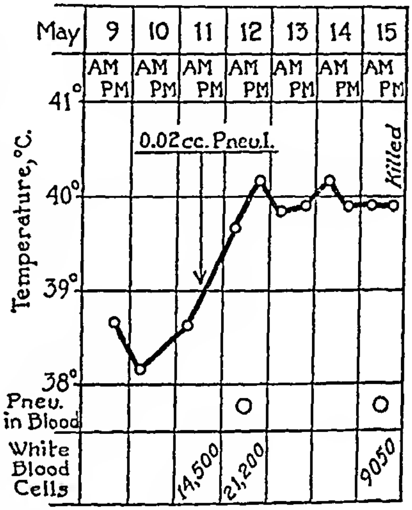
† This dog had had two previous attacks of experimental lobar pneumonia.

The lower part of the metastatic lesion, right upper lobe, showed a well developed macrophage response with beginning clearing and disappearance of pneumococci while the upper part of the lobe was characterized by a diffuse early inflammatory process containing great numbers of extracellular pneumococci often present in masses.

Dog 9S exhibited in general the same picture in the initial clearing lesion, but the macrophage reaction was distributed throughout the lesion in foci of varying size. The sterile culture obtained from the



TEXT-FIG. 1



TEXT-FIG. 2

TEXT-FIG. 1. Dog 9H. Infected with 0.02 cc. culture of pneumococcus Type I in the right lower lobe. Developed lobar pneumonia involving right lower, right middle, left middle and left upper lobes in progression.

TEXT-FIG. 2. Dog 9S. Infected with 0.02 cc. culture of pneumococcus Type I in the right lower lobe. Developed lobar pneumonia involving right lower, middle and upper lobes progressively.

posterior part of the lobe probably represented aspirated exudate from such an area of macrophage mobilization.

The number of pneumococci observed in the inflammatory processes was roughly in inverse proportion to the development of the macrophage reaction. With one exception, dog 69L, lesions showing this cellular change in its pronounced form, were sterile in culture, and in this animal no intact pneumococci were observable microscopically.

However, the disappearance of pneumococci was not uniformly confined to those lesions exhibiting a macrophage reaction. In one instance a 48 hours old lesion (left middle lobe of dog 9H) characterized by a predominantly polymorphonuclear cellular exudate, yielded a sterile culture. Only a very few intracellular pneumococci were seen in the sections. In two other metastatic processes (dogs 100R and 69L) pneumococci were few and all intracellular in the areas of polymorphonuclear exudate. It will be noted in the table that all three of these lesions showed a beginning though slight focal macrophage reaction.[1]

Humoral Immune Substances.—No evidence of acquired antipneumococcal humoral immunity was found in any of the five animals tested. The heated serum, secured just before death, failed to protect mice against as few as 10 pneumococci. In one dog, 59S, tests of the natural pneumococcidal-promoting activity of the blood serum were made daily throughout the course of the disease. Before infection the fresh serum of this animal mixed with normal dog leucocytes caused the destruction of approximately 10,000 pneumococci and continued to show this degree of activity until the time of death.

DISCUSSION

Evidence of local recovery of the pulmonary lesion is occasionally observed in clinical lobar pneumonia and has been designated in the literature as migrating pneumonia. We have studied several such cases, one of a particularly striking nature, in which practically complete resolution of the original right lower lobe lesion occurred during a spread to the upper lobe of the same side between the 5th and the 8th days of the febrile course (4). Another patient showed an initial consolidation of the lateral third of the left lower lobe which gradually spread medially to include the whole lobe. During the course of the extension the distal part of the lesion exhibited progressive clearing until on the 4th day the x-ray shadow was confined to the medial half of the lobe. Numerous other instances have been observed in which late in the course of the disease the x-ray showed clearing of the initial lesion and increasing density of a recently consolidated lobe.

[1] The degrees of macrophage reaction have been illustrated in a preceding paper (1).

These changes observed during the course of lobar pneumonia in the human being together with the not infrequent finding of whole lobes in a state of resolution at post mortem are essentially the same as those seen in the experimental canine disease and provide additional evidence for the similar nature of the two infectious processes.

Local recovery during the active stage of infection is by no means confined to pneumonia. Certain other infectious diseases exhibit this phenomenon much more frequently and in several conditions it constitutes one of the characteristic features of the disease. Among those most commonly observed are staphylococcus and streptococcus infections of the skin, notably erysipelas. Rheumatic fever in its typical form would fall into this category, and probably many of those infections in which so called complications due to the same etiological agent develop during or subsequent to the subsidence of the primary lesion. Healing and spreading lesions are not uncommonly observed in pulmonary tuberculosis. This list is not exhaustive. Unfortunately there are not many detailed data available on the nature of the immune reactions of the healing tissues in most of the above mentioned conditions. However, one of these, erysipelas, has been investigated extensively by Birkhaug (5), Gay (6) and others. The healing lesion in this condition is characterized by the mobilization of macrophages which as Gay and his coworkers (7) have shown are highly effective in the disposal of hemolytic streptococci, much more so than are the polymorphonuclear leucocytes.

The present study of what might be termed interrupted recovery[2] throws further light on the process by which the body terminates the pneumonic infection. It would seem likely that the mechanism which produces early recovery in a part of the lung is the same as that which finally brings the infection under control and ends the disease. There are no essential histological differences between the lung lesion at recovery and the lesions showing resolution before this time. The absence of detectable acquired humoral immune substances in the great majority of dogs at the time of recovery is another point of similarity. However, the question arises as to whether the very occurrence of a metastatic lesion at the time of healing of the original

[2] Our observations on a large number of dogs with experimental lobar pneumonia indicate that all six of the dogs dealt with in this study would recover.

focus of infection does not indicate the absence of some factor which appears at recovery. While no direct answer to this question can be given there are certain experimental data bearing on the subject. A study of recurrent attacks of pneumonia experimentally produced in the dog (8) showed that pneumonic lesions could be induced repeatedly with doses as small as those employed in the primary infection. In a more recent investigation it was found that the production of a high degree of general immunity both active and passive did not prevent the inception of a pneumonic lesion following experimental infection (9). These findings suggest that when a suitable inoculum of pneumococci is implanted in the terminal air spaces[3] even the highly pneumococcus-resistant body is not capable of inhibiting the local multiplication of the microorganisms. However, the difference between such secondarily induced lesions and the initial ones is the brief duration of the former, which often became sterile within 24 hours or less. The late metastatic lesions occurring in the dogs reported in this investigation resemble those produced in immune animals as shown by the sterile cultures obtained from parts of the consolidated lobe in two instances. In several other dogs showing this phenomenon of simultaneous clearing and spread and which were not sacrificed, recovery occurred within a day or two after the appearance of the metastatic lesion. Another point of resemblance between these two types of lesions is the early appearance of the macrophage reaction. In the primary lesions of artificially immunized dogs as well as in the secondarily induced pneumonic processes of recovered animals a macrophage reaction occurred usually within 24 hours after the inception of the infection. Similarly four of the six 24 hour metastatic lesions shown in Table I exhibited a macrophage response in some part of the recently developed inflammatory process.

The findings discussed in the above paragraph suggest that the dog showing beginning resolution of the initial lesion has already acquired increased antipneumococcal resistance. Is such immunity principally local or general in character? On the basis of an extensive study of the macrophage reaction occurring in the pulmonary lesions

[3] The method of producing experimental lobar pneumonia by the intrabronchial injection of pneumococci suspended in starch results in the filling of many alveoli by the inoculum.

of dogs sacrificed at varying stages of experimental pneumonia or dying spontaneously from the disease it was inferred that the mechanism of recovery is of dual nature, consisting of a general process which acts to localize the infection and prevent or control invasion of the blood stream and a local process by which the lungs are ultimately freed of the invading microorganisms (1). If both processes are active recovery results. If either one or the other fails, the infection terminates fatally. Concerning the nature of the general process our information is by no means complete. That it consists in part of the pneumococcidal activity of the blood is indicated by the persistence of this property in infected animals showing little or no bacteremia, and its usual disappearance when blood invasion becomes pronounced.[4] The absence of detectable acquired immune bodies in the dogs showing clearing and spread of the pneumonic lesion together with lack of any increase in the natural pneumococcus-killing power of the blood in the one dog of this series and in a number of other recovering animals tested daily throughout the course of the disease suggests the lack of any augmentation of this type of immunity at the time of recovery. Determination of the clearing power of the blood might throw light on this problem since it would provide evidence of the functional activity of the reticulo-endothelial cells of the body. However the interpretation of such tests would be difficult because of the great variability in the natural resistance of dogs to pneumococcus infection.

While the lack of any detectable changes in the general process of immunity does not exclude the occurrence of some unknown factor at the time of recovery it does accentuate the importance of the local cellular changes which are observed to be directly associated with the disappearance of pneumococci from the lesion. The early appearance of the macrophage reaction in the young metastatic lesions suggests an increased reactivity of the fixed tissue cells of the lungs to the pneumococcus. Whether this is principally local in nature or is a local manifestation of a more general altered cellular reaction is not clear. Former experiments on the reinfection of dogs recovered from an

[4] Exceptions to this finding were discussed in the former communication (1).

attack of lobar pneumonia showed that a second infection produced in a part of the lung not previously affected, was characterized by an earlier macrophage response than was observed in primary lesions, although definitely less prompt than when the lesion was induced at the site of the preceding one. This would suggest some general effect on the lung cells. However, unreported experiments[5] on the injection of killed pneumococci into cantharides blisters produced in patients after recovery from lobar pneumonia revealed no more rapid accumulation of macrophages than occurred in normal individuals.

The subject of local immunity of the lung in experimental pneumococcus pneumonia will be dealt with in a subsequent communication.

SUMMARY

An investigation has been made of the process of local recovery occurring in the pulmonary lesions of dogs with experimental pneumococcus lobar pneumonia. Six animals showing simultaneous healing and spread of the pathological process in different parts of the lungs were sacrificed during the active stage of the disease for bacteriological and histological study. It was found that with few exceptions the clearing (resolving) lesions as revealed by x-ray during life were sterile on culture while the young, metastatic processes yielded an abundant growth of pneumococci. The resolving areas which represented the older lesions were characterized by the presence of a well developed macrophage reaction, whereas the early lesions consisted of alternating areas of edema and polymorphonuclear infiltration and contained usually many pneumococci both free and in the cells. The only pneumococci found in the areas of macrophage mobilization were intracellular forms, for the most part in the process of digestion. In several instances these striking differences in the cellular picture and the numbers and distribution of pneumococci were observed in different parts of a single lobe lesion. Tests on the blood serum failed to reveal any evidence of acquired antipneumococcal humoral immunity. The significance of these findings in relation to the mechanism of recovery is discussed.

[5] Carried out by Dr. W. D. Sutliff.

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EXPLANATION OF PLATES

Plate 24

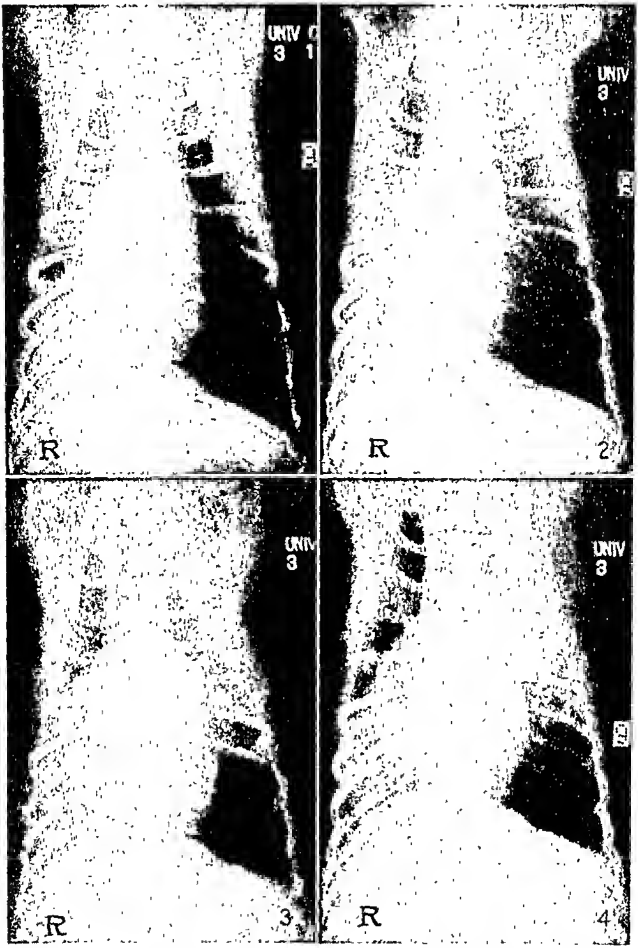
Figs. 1 to 4 are x-rays of dog 9H taken at intervals of 24 hours until the dog was sacrificed at 96 hours.

Fig. 1. Consolidation of the right lower lobe at 24 hours after the inception of the disease.

Fig. 2. By 48 hours the pulmonary lesion has extended upwards to involve the right middle lobe.

Fig. 3. A further spread of the pathological process to the left middle lung field is shown in the x-ray taken at 72 hours. There is a questionable slight clearing of the initial lesion at this time.

Fig. 4. At the end of 96 hours the new lesion in the left side has extended to involve the entire upper half of the lung field and definite clearing of the right side lesions is now apparent.



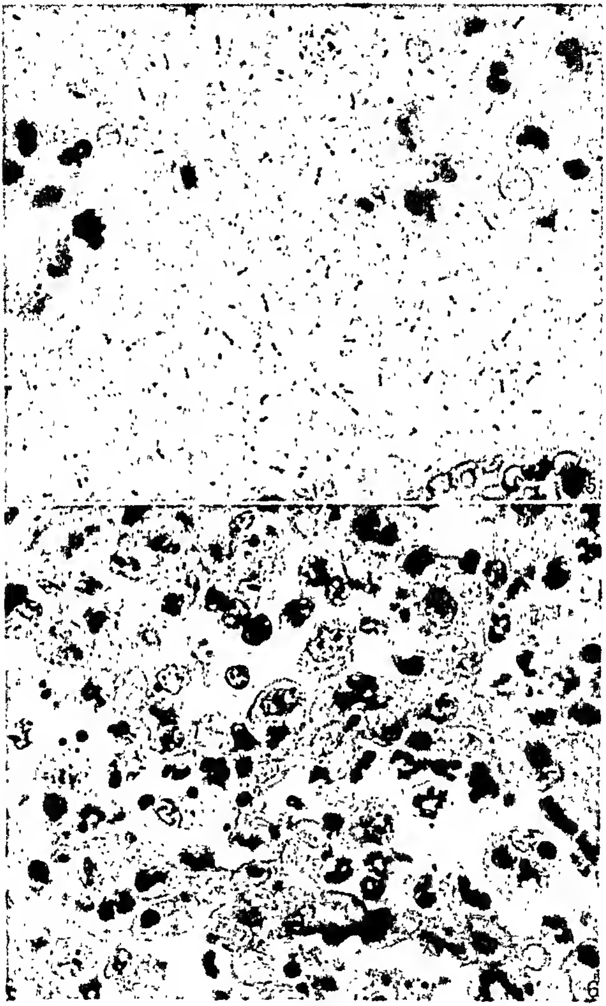
(Robertson and Coggeshall: Local recovery in pneumococcus pneumonia)

PLATE 25

Sections fixed in Zenker-acetic solution, mounted in paraffin and stained with Wallace's modification of the Gram-Weigert stain.

FIG. 5. Dog 9H. Section of early spreading lesion in left upper lobe showing large numbers of pneumococci free in the exudate which consists of edema fluid and a few polymorphonuclear leucocytes. × 1000.

FIG. 6. Dog 9H. Section of the initial lesion in right lower lobe (sterile on culture) which shows a marked macrophage reaction with disappearance of pneumococci and beginning resolution. The alveoli are partially collapsed. The septa are much thickened due to the presence of large mononuclear cells in them. The exudate contains many macrophages, some of which are seen to be still attached to the alveolar walls. × 1000.



(Robertson and Coggeshall: Local recovery in pneumococcus pneumonia)

JAPANESE B ENCEPHALITIS VIRUS: ITS DIFFERENTIATION FROM ST. LOUIS ENCEPHALITIS VIRUS AND RELATIONSHIP TO LOUPING ILL VIRUS

By LESLIE T. WEBSTER, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

PLATES 26 AND 27

(Received for publication, January 12, 1938)

When encephalitis broke out in August and September of 1932 and 1933, centering in Illinois and Missouri, it was said to resemble Japanese B summer encephalitis (1). But after the St. Louis virus agent was discovered and found to be neutralized specifically by sera from convalescents, additional tests showed that this virus was not neutralized by sera from convalescents of Japanese B encephalitis (2). Further comparisons could not be made at that time because the etiological agent of Japanese B encephalitis had not yet been isolated, but the two diseases appeared to be immunologically distinct (2).

Japanese encephalitis recurred in epidemic form in 1934 and 1935, and from a number of cases virus was recovered. Hayashi, in 1934, reported the transmission of a virus from brain tissue of a fatal case to monkeys for several generations (3), and in 1935, October to December, Kawamura, Hashimoto, Kasahara, Kaneko, Takaki, Taniguchi, and Mitamura reported successful inoculations of brain tissue from fatal cases into mice, and occasionally into monkeys (4). Supplementing these early statements, further reports became available, in which the virus was related directly to the human disease (5–8).

The Japanese workers regarded their virus as similar in many respects to the St. Louis virus. Hence an exchange of Japanese and St. Louis strains and sera was effected between Drs. Kodama, Hashimoto, Takaki, Kasahara, and Mitamura and ourselves in the spring of 1936, and comparative studies were continued in Japan and in our laboratories.

More recently available reports from Japan (9–13) agreed that the

strains of Japanese virus recovered by various workers are identical; that they were neutralized by sera of a large percentage of tested convalescents and by sera of a few individuals without a history of encephalitis living in the epidemic areas, but not by sera of persons without encephalitis living in regions free of the disease (10, 11). Finally, they regarded the Japanese virus as generally similar but not identical with the St. Louis virus (9–11, 13).

The onset of the experimental disease in mice was said to be more frequently accompanied by paralysis of the posterior extremities (8, 9, 5, 7), and the virus following nasal instillation had a greater tendency to enter the blood stream (9) and a greater virulence when injected intraperitoneally (8, 9, 5, 7). In monkeys the virus was more virulent in that it induced a rapid and fatal cerebellar syndrome (9).

Kawamura and associates (9), and Kasahara and associates (13) found no cross-protection in hyperimmune Japanese and St. Louis rabbit sera nor in sera of convalescents of the two diseases. Kudo (10) and Takaki (11) observed no cross-protection of St. Louis hyperimmune rabbit sera but some crossing of Japanese immune rabbit sera.

The present report of our own studies on Japanese encephalitis, besides confirming for the most part those of the Japanese workers, shows certain relationships between this and other viruses associated with epidemic encephalitis in man.

Characteristic Reactions of Japanese Virus in Animal Species

The Japanese virus[1] induced reactions in animal species which were readily distinguished from those produced by St. Louis virus but approximated closely those of louping ill virus. It proved innocuous in rabbits and guinea pigs but induced in mice, monkeys, and sheep a fatal encephalitis.

Thus Swiss mice developed encephalitis following injections of virus by the intracerebral, intranasal, subcutaneous, and intraperitoneal routes. Following nasal instillation they showed, after 5 to 8 days, paralyses of the posterior extremities, or occasionally tremors and convulsions. They became prostrate and died in 7 to 10 days. The experimental disease resembled that following inoculation

[1] Six strains received from five Japanese investigators proved similar in all respects as tested in our laboratory. They passed Seitz filters readily, were virulent when inoculated in 0.03 cc. amounts intracerebrally into Swiss mice to the 10^{-7} dilution, and when frozen and dried retained their virulence well.

with louping ill virus and with St. Louis virus except for a more frequent onset with paralysis. Histological examination of tissue of these mice showed a type and distribution of lesions similar to those produced by St. Louis and louping ill viruses. The brain showed perivascular and subdural accumulations of round cells plus specific necrosis of nerve cells in the olfactory tracts, Ammon's horn (Fig. 1, and Kawamura, Fig. 4 (9)), anterior limbic area, hypothalamus, and, at late stages, throughout the cortex. The spinal cord remained relatively normal. Virus injected intraperitoneally or even subcutaneously in relatively small doses, 100 to 1,000 times the minimum intracerebral dose, usually induced encephalitis. Louping ill virus was similar in this respect but St. Louis virus was innocuous by these routes unless massive doses were employed. Moreover, Japanese and louping ill viruses, inoculated subcutaneously or intraperitoneally, reached the circulating blood promptly and persisted longer than St. Louis virus. Finally, Japanese and louping ill (14) viruses, following nasal instillation, were readily recovered from the blood stream, while St. Louis virus was rarely found.

Macacus rhesus monkeys were susceptible to the Japanese virus inoculated intracerebrally (8, 9, 5, 7) or intranasally. Following nasal instillation they showed an elevation of temperature to 106° on the 4th day, and a severe cerebellar ataxia on the 5th or 6th day. They became prostrate and died within 10 days. Samples of blood drawn daily and injected intracerebrally into mice failed to show virus. Sections taken at autopsy showed lesions limited primarily to the brain and consisting of necrotic nerve cells scattered irregularly, plus foci of round cells surrounding small blood vessels. Necrosis of the Purkinje cells of the cerebellum was especially marked (Fig. 2, and Kawamura, Fig. 14 (9)). These cells in the superficial convolutions appeared enlarged, or with pycnotic nuclei and granular cytoplasm, or shrunken with deep staining cytoplasm. In the deeper crypts many of the Purkinje cells were entirely missing and the few remaining were small and distorted. Nearby, a local "gliosis" was not uncommon. There was very little change in the surrounding tissue. The reactions described above following nasal instillation are generally similar to those following intracerebral injection of the virus. They are also similar to those following intracerebral inoculation of the louping ill virus (15). Monkeys injected with St. Louis virus, on the other hand, either remained normal or developed a mild, non-fatal encephalitis. Lesions could not be demonstrated with certainty without multiple intracerebral injections.

Sheep inoculated intracerebrally or intranasally with Japanese virus developed an acute, fatal encephalitis.

Lambs weighing 40 to 60 pounds received an intranasal instillation of 1 cc. of the mouse brain virus diluted 1 to 100. Temperatures rose on the 4th day to 106.0°, the animals became quiet, lost appetite, their heads drooped and legs weakened, and by the 8th day they were unable to rise. No definite central nervous system signs were noted. Blood drawn daily and injected intracerebrally into mice showed no virus. At autopsy, brain tissue injected into mice brought them down promptly with the characteristic signs of the Japanese disease. Sections of brain tissue showed blood vessels generally engorged and surrounded by

many round cells. Foci of round cells were likewise scattered irregularly throughout the brain. Many Purkinje cells of the cerebellum were in various stages of necrosis and neighboring glial cells appeared abnormal (Fig. 3). Scattered nerve cells throughout the cortex and brain stem were likewise necrotic. As with monkeys, the reactions described above following nasal instillation are generally similar to those following intracerebral injection of the virus. They are also similar to those following intranasal and intracerebral inoculation of the louping ill virus (Fig. 4).

1 cc. of virus diluted 1 to 10 and injected subcutaneously into lambs proved harmless but immunized them against a later nasal injection fatal to unvaccinated controls.

TABLE I

Virulence of Central Nervous System Viruses of Man Inoculated Intracerebrally into Laboratory Animals

Virus	Rabbit, Guinea pig	Mouse	Macacus monkey	Young sheep
Rabies	++	++	++	++
Louping ill; Japanese B encephalitis; Australian X (?)	0	++	++	++
St. Louis encephalitis	0	++	0–±	0
Poliomyelitis	0	0	+	0

These reactions in animal species differentiate the Japanese B from the St. Louis virus but relate it closely to louping ill virus.

A presumptive differential diagnosis of Japanese B encephalitis and other viruses associated with a primary encephalitis of man may be carried out on the basis of the above reactions in animal species in the manner outlined in Table I.

Lack of Cross-Resistance of Immunized Mice

Mice immune to Japanese virus were not immune to St. Louis virus, and conversely, mice immune to St. Louis virus were not immune to Japanese virus. Tests were not made with louping ill virus.

Mice were immunized against Japanese and St. Louis virus by repeated subcutaneous injections of sublethal doses of the virulent homologous virus. Attempts to immunize mice against louping ill virus failed. Sublethal doses, even when repeated, did not immunize while larger doses, lethal to 20 to 30 per cent of vaccinated mice, left a selected group of survivors whose subsequent resistance

to homologous or heterologous virus could not be judged as due to specific immunity factors alone, but might have depended on initial, non-specific, inherited factors. For this reason these survivors were not considered proper material for testing and were discarded.

The Japanese and St. Louis virus mice were tested after 2 to 4 weeks with homologous and heterologous virus in graded intracerebral doses. Unvaccinated mice were likewise tested as controls. The protocol of one such experiment is shown in Table II, in which the Japanese virus proved fatal to unvaccinated and to St. Louis vaccinated mice through the 10^{-6} dilution but not to mice immunized with Japanese beyond the 10^{-5} dilution. Similarly, the St. Louis virus was fatal alike to unvaccinated and Japanese virus mice through the 10^{-6} dilution but not to St. Louis virus mice beyond the 10^{-4} dilution.

TABLE II

Absence of Cross-Resistance of Mice to Japanese B and St. Louis Encephalitis Viruses

Mice vaccinated against	Vaccinated mice tested with	Duration of life of tested mice in days		
		0.03 cc. of test virus diluted		
		10^{-5}	10^{-6}	10^{-7}
Unvaccinated controls	St. Louis virus	*5, 5, 5, 5	5, 5, 5, 6	†S, S, S, S
St. Louis virus	" " "	S, S, S, S	S, S, S, S	—
Japanese B virus	" " "	6, 6, 6, 7	5, 6, 6, 7	—
Unvaccinated controls	Japanese B virus	5, 5, 5	6, 6, 6, 7	7, 7, 8, S
Japanese B virus	" " "	8, 8, 8, 9	S, S, S, S	—
St. Louis virus	" " "	5, 5, 5, 5	7, 8, 9, S	—

* Mouse died of encephalitis 5 days following injection.

† Mouse remained well following injection. Discarded at 30 days.

Lack of Cross-Protection of Immune Sera

Protection tests disclosed no definite immune relation between Japanese B, St. Louis, and louping ill viruses.

Tests with hyperimmune sera were made in the following manner.

Monkeys were each given repeated subcutaneous and intraperitoneal injections of the respective living mouse brain viruses. Sera were drawn and mixed undiluted with various concentrations of mouse brain virus prepared in the usual manner in broth as a diluent. After standing at 37°C. for 2 hours and at 23°C. for 2 hours, the mixtures were each injected intracerebrally in 0.03 cc. amounts into four Swiss mice. Duration of life of the injected animals was recorded in days.

TABLE III

Lack of Cross-Neutralization of Japanese and St. Louis Encephalitis, and Louping Ill Viruses in Heterologous Sera

Test sera		Test virus	Duration of life of tested mice in days. Dilution of virus in serum mixtures. 0.03 cc. injected			
			10^{-4}	10^{-5}	10^{-6}	10^{-7}
Monkey	Normal	Japanese No. 2	*5, 5, 5, 7	7, 7, 8, 8	8, 8, 9	7, 7, 12
"	Japanese No. 2 immune	" " "	9, 11	S, S, S, S	S, S, S	—
"	St. Louis immune	" " "	—	7, 7, 8, 10	7, 8, 10	—
"	Louping ill immune	" " "	—	8, 9, 10, S	10, 11, S	—
"	Normal	St. Louis	—	5, 5, 7, 7	10, 10, 10, 11	
"	St. Louis immune	" "	7, 7, 9	S, S, S, S	—	—
"	Japanese No. 2 immune	" "	—	9, 9, 10, 10	S, S, S, S	
"	Louping ill immune	" "	—	7, 9, 9, 9	11, 11, S, S	
"	Normal	Louping ill	7, 7, 7, 7	7, 8, 8, 8	9, 9, 9, 10	8, 9, 9
"	Louping ill immune	" "	S, S, S, S	S, S, S, S	S, S, S, S	—
"	Japanese No. 2 immune	" "	—	7, 8, 9, 9	9, 9, 11, 11	
"	St. Louis immune	" "	—	9, 9, 9, 9	8, 9, 9, 9	

S = mouse remained well 21 days.

— = dilution not tested.

* Mouse died of encephalitis 5 days following injection.

The protocol summarized in Table III shows Japanese virus No. 2 plus normal monkey serum fatal to 50 per cent or more of four mice per dilution through 10^{-7}, fatal through 10^{-4} when combined with homologous immune serum, and through 10^{-6} when mixed with St. Louis and louping ill immune sera respectively. Similarly, St. Louis virus mixed with normal monkey serum was fatal through the 10^{-6} dilution, with homologous immune serum through the 10^{-4} dilution, and with Japanese or louping ill, through the 10^{-5} and 10^{-6} dilutions respectively. Finally, louping ill virus mixed with normal monkey serum was fatal through the 10^{-7} dilution, with homologous immune serum, in less than the 10^{-4} dilution, and with Japanese and St. Louis sera through the 10^{-6} dilutions, respectively. In short, virus mixed with heterologous immune sera, although slightly less active than with normal monkey serum, was 10 to 100 times more active than when combined with homologous immune sera.

Sera from St. Louis convalescents did not protect against the Japanese virus nor did sera from Japanese convalescents protect against St. Louis virus. A curious instance of cross-protection, however, has been noted by Kuttner and confirmed in our laboratory.

Kuttner described two cases of encephalitis contracted by Europeans in China (16). Serum from W. protected against both Japanese and St. Louis viruses, and serum from G. protected against Japanese but was not tested against St. Louis virus. Further sera from these cases[2] were sent directly to our laboratory and were found to protect well against both Japanese and St. Louis and not against louping ill virus.

Finally, sera from Japanese and St. Louis convalescents did not protect against louping ill virus nor did sera from supposed convalescents of louping ill virus (17) protect against Japanese or St. Louis viruses.

DISCUSSION

The virus from Japan described above is regarded as the etiological agent of Japanese B summer encephalitis on the following grounds. During an epidemic outbreak, strains were recovered from brain tissue of fatal cases of encephalitis by several independent investigators in Japan at the same time and by the same technique. All strains have proved identical in so far as tested. They were said to be neutralized specifically by sera from convalescents and certain contacts. The virus proved similar to others in the encephalitis group

[2] Obtained through the kindness of Dr. Kuttner.

and yet ultimately distinct. Japanese B encephalitis is included, therefore, in the group of primary central nervous system infections of man of known virus etiology which occur in epidemic form in late summer. These infections, poliomyelitis, Japanese B encephalitis, Australian X disease, and St. Louis encephalitis, have features in common but are distinguishable by laboratory tests. Outbreaks of each are frequent and limited chiefly to hot weather, and cases of each are scattered throughout an infected community, usually not more than one per family. Clinically the diseases are often difficult to identify. Each may be recognized, however, by testing sera of convalescents for specific neutralizing properties against one of these virus agents, or by obtaining virus from brain tissue of fatal cases and testing its virulence for mice, monkeys, and sheep and its neutralization in specific antisera.

Possible relationships between Japanese B encephalitis, the sheep encephalitis of Scotland (louping ill), and polioencephalitis of children in Australia (X disease) should be further explored. The sheep virus is probably infectious for man, judging from the fact that three investigators, shortly after commencing work with the virus (17), contracted encephalitis and later showed specific neutralizing antibodies in their sera. Moreover, louping ill virus has been related to the virus associated with X disease of children in Australia (18), and now has proved similar to the newly discovered Japanese B encephalitis virus. Further studies are needed to determine whether this relationship is merely superficial or of immediate epidemiological importance.

The question of mode of spread of this group of infections through a community and their portal of entry into the body are unknown and difficult to investigate. Laboratory tests on susceptible animals indicate clearly that the most vulnerable portal for the experimental introduction of these viruses is the nasal mucosa: this is the route of choice in mice for the St. Louis virus; in mice, monkeys, and sheep for the Japanese and louping ill (21) viruses; and in monkeys for the poliomyelitis virus. There is also evidence that spontaneous louping ill may occur both in man (17) and in mice (20) through the nasal mucosa. But contact experiments to induce spontaneous transfer of St. Louis, Japanese B, and poliomyelitis infections among susceptible

laboratory animals have failed and it is difficult or impossible to detect the virus in the upper respiratory tract of the diseased individual. On the other hand, subcutaneous injections of Japanese B and louping ill viruses in mice and of poliomyelitis virus in monkeys and possibly man may also induce disease. Moreover, reports state that in nature louping ill infection of sheep takes place subcutaneously by the bite of an insect (19). But experiments to detect virus in the blood of individuals following natural or experimental infection are either positive for brief periods only (louping ill, Japanese B viruses) or are completely negative (poliomyelitis, St. Louis viruses). Hence there is evidence for and against both the upper respiratory and subcutaneous routes of transmission of these infections and the question remains perplexing.

CONCLUSIONS

1. Japanese B encephalitis virus, obtained from Japanese investigators, has proved virulent for mice and monkeys, confirming the reports from Japan. It has also been found virulent for monkeys when instilled intranasally and for sheep when introduced intracerebrally or intranasally.

2. Japanese B encephalitis virus has been differentiated from St. Louis virus and found similar to louping ill virus according to its reactions in animal species. Serologically, however, it is distinct.

3. Japanese B encephalitis and its related group of primary virus encephalitides of man have been discussed with regard to their differentiation and mode of spread.

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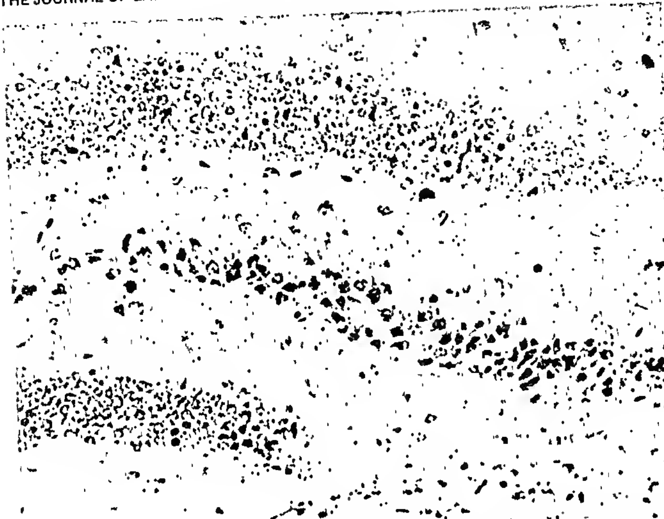
EXPLANATION OF PLATES

PLATE 26

FIG. 1. Swiss mouse. Section through Ammon's horn 6 days after nasal instillation of Japanese B encephalitis virus. The left and right vertical columns of pyramidal cells appear relatively normal. Cells in the central column are in various stages of necrosis. Eosin-methylene blue. × 275.

FIG. 2. Macacus monkey. Section through cerebellum 8 days after nasal instillation of Japanese B encephalitis virus. The Purkinje cells are necrotic or entirely absent. Eosin-methylene blue. × 275.



1

2

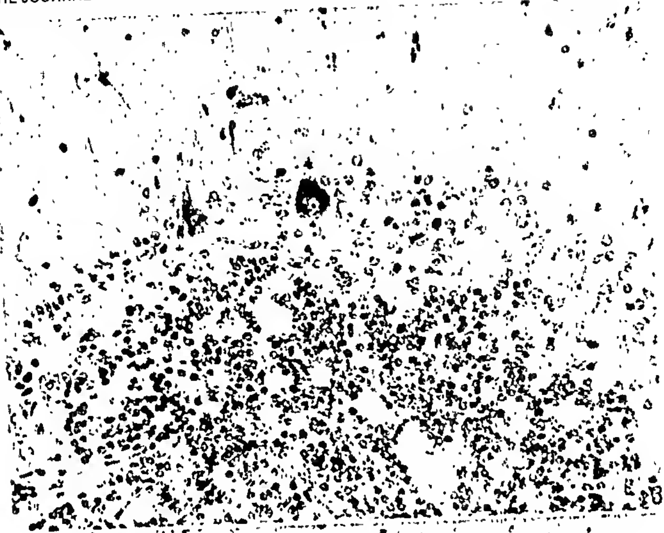
Photographed by Joseph B. Haulenbeek

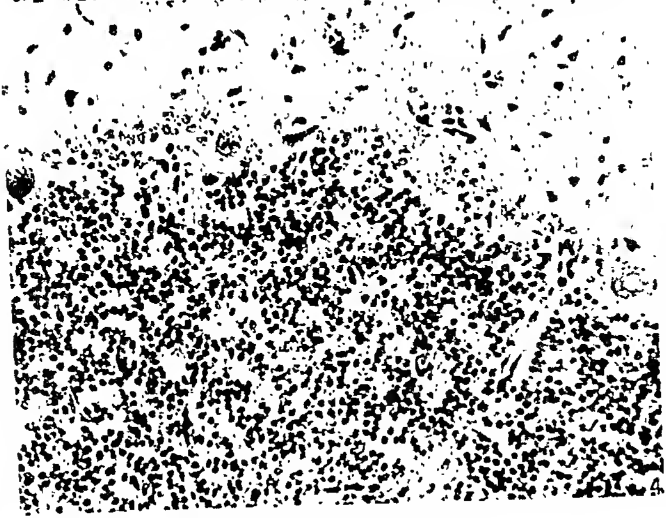
B encephalitis virus)

PLATE 27

FIG. 3. Young sheep. Section through cerebellum 9 days after nasal instillation of Japanese B encephalitis virus. The Purkinje cells are in early stages of necrosis. A few are missing entirely. There is some reaction of glial cells in the molecular layer. Eosin-methylene blue. × 275.

FIG. 4. Young sheep. Section through cerebellum 8 days after nasal instillation of louping ill virus. The Purkinje cells are necrotic and there is reaction of neighboring glial cells. Eosin-methylene blue. × 275.





Photographed by Joseph B. Haulenbeck

THE INFLUENCE OF INFLAMMATION ON THE ABSORPTION OF SUBSTANCES OF VARIED DIFFUSIBILITY*

By ROSE G. MILLER, Ph.D.

(*From the Department of Pathology, Cornell University Medical College, and New York Hospital, New York*)

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It has long been recognized that diffusible substances are absorbed from the tissues by direct entrance into the blood vessels. Dandy and Rowntree (1) made a quantitative study of absorption from the peritoneum. They found that when the diffusible dye, phenol red, is injected intraperitoneally, 40 to 60 per cent is excreted in the urine during the first hour after injection, while less than 0.1 per cent enters the thoracic lymph during this time. On the other hand, colloidal solutions and particles in suspension depend largely on the lymphatic system for absorption. Lewis (2) injected horse serum subcutaneously into a dog; using the complement fixation reaction, he was able to detect horse protein in the thoracic lymph 40 minutes after injection, while only after 3 hours did any protein appear in the blood. Drinker and Field (3) also found that horse serum is absorbed largely by way of the lymphatics. However, it should be mentioned that certain more diffusible colloidal solutions may enter the blood vessels directly. Bolton (4) found that the absorption of colloidal dyes of relatively small molecular dimensions is accomplished both by the vascular and by the lymphatic systems, but these colloidal dyes diffused into the blood more slowly than crystalloids.

Previous studies of the removal of substances from inflamed areas have dealt largely with the dissemination of bacteria. Noetzel (5) and Pawlowsky (6) found that when bacteria are injected into a joint they soon appear in the blood stream, but if the bacterial injections were made into a joint which had previously been treated by the injection of a sterile irritant, the dissemination of the bacteria was greatly inhibited. Hoehne (7) and Opie (8) found that if bacteria are injected into an inflamed peritoneal cavity, their entrance into the blood stream may be partially or completely inhibited.

Interest in the absorption of soluble substances from inflamed areas was aroused by the experiments of Opie on the Arthus phenomenon. Opie (9) demonstrated that when protein is injected into the skin of a specifically immunized animal, it remains at the site of injection, where contact of antigen and antibody produce an

* This paper is part of a thesis submitted to the Graduate Faculty of Cornell University in partial fulfillment of the degree of Doctor of Philosophy.

acute inflammatory reaction; and the injected protein does not enter the circulating blood. Menkin (10) extended these observations to a study of non-specific inflammation. He found that trypan blue, horse serum and colloidal iron are absorbed more slowly from inflamed than from normal areas. Okuneff (11) has presented corroborative data with trypan blue.

Few reports appear in the literature on the absorption of diffusible substances from inflamed areas. Underhill, Kapsinow and Fisk (12) concluded that phenol red was absorbed more slowly from burned areas than from normal areas. On the other hand Hudack and McMaster (13) found that inflammation facilitated the absorption of a diffusible as well as of an indiffusible dye. The object of the present experiments was to study the rate of absorption of soluble substances of varied diffusibility from inflamed areas.

General Methods

The substances used may be classified as proteins, carbohydrates and dyes. Horse serum globulin and crystalline egg albumin were the proteins studied. The carbohydrates were glucose and the pneumococcus Type I specific polysaccharide. The dyes were trypan blue, phenol red and brom phenol blue. These substances were chosen because they are relatively stable, non-toxic compounds, which can be detected readily by chemical or immunological methods. Furthermore, none of them (with the exception of several of the dyes) is known to enter into ready combination with protein under the conditions of the experiments. The effect of the combination of phenol red with protein is discussed under the experiments performed with that substance.

Production of Inflammation.—Male rabbits weighing 1600 to 2300 gm. were used. Animals of approximately the same weight group were used in each experiment. The inflammatory irritants employed were 5 per cent aleuronat and 3 per cent starch solution in 0.5 per cent saline, living avirulent staphylococcus cultures, staphylococcus vaccines, and in a few experiments broth which had been concentrated 20 times. The staphylococcus vaccines were prepared by heating 24 hour broth cultures of staphylococci at 60°C. for 30 minutes.

Great variation was found in the response of different animals to the same irritant. 24 hours after the injection of the irritant into the peritoneal cavity there was hyperemia and slight roughening of the peritoneal surfaces. No free fluid was present in the cavity. Shreds of fibrin were sometimes seen. Larger variation occurred in the subcutaneous inflammations. In general, these reactions were visible, 4 to 6 hours after the injection of the irritant, and consisted of swelling and induration of the skin, measuring about 4 to 7 cm. in diameter. On section there were marked edema and redness of the subcutaneous tissues, and to a lesser extent of the underlying muscle. Occasionally, a thin layer of pus

appeared under the skin at the end of 48 hours. Abscess formation occurred in only one experiment. The greatest variations were found in the cutaneous inflammations. The reaction to the staphylococcus injections was generally visible 2 to 6 hours after the injection, and consisted of a swollen, pale or erythematous area measuring about 1.5 to 4.0 cm. in diameter. Sometimes there was a hemorrhagic center surrounded by a pale area which in turn was surrounded by a zone of hyperemia. At other times a small necrotic focus appeared in the center of the injected area. The reactions produced by the injection of concentrated broth consisted of thickened erythematous areas measuring 1.5 to 2.5 cm. in diameter.

The Influence of Inflammation on the Absorption of Proteins

Methods.—Horse serum globulin was prepared by the addition of saturated ammonium sulfate to an equal quantity of horse serum. The resulting precipitate was centrifuged and dissolved in dilute sodium chloride solution. It was reprecipitated, and dialyzed against cold running tap water. No attempt was made to separate euglobulin from pseudoglobulin. Crystalline egg albumin was prepared by the method of Hopkins and Pincus (14). The egg albumin was recrystallized and dialyzed until the dialysate contained no further trace of ammonium sulfate. The protein solutions were sterilized by filtration through Berkefeld N or Seitz filters. The final dilution fluid contained 0.5 per cent salt solution. Protein content was determined by the method of Shevsky and Stafford (15) and was checked occasionally by Kjeldahl determinations.

Antisera were produced by the repeated intravenous and subcutaneous injections of the protein solution into rabbits. The titers obtained were 1–50,000 to 1–200,000 of globulin and 1–10,000 to 1–40,000 of egg albumin. Precipitin tests were performed by the usual antigen dilution method. 0.3 cc. of antiserum was added to 0.3 cc. of the diluted antigen. The tubes were allowed to stand at room temperature for 2 hours and then placed in the ice box overnight. Readings were made the following morning.

The attempt was made to determine if horse serum globulin disappeared from the site of inflammation in the skin. Rabbits were injected intracutaneously with an inflammatory irritant, for example, *Staphylococcus aureus*. The area of skin chosen was the flank about 4 cm. above the inguinal lymph node. 0.2 cc. of a 2 to 3 per cent globulin solution was injected into the inflamed area and into a similar control area on the opposite side. From 5 to 24 hours after the injection of the globulin, the animal was killed and the injected sites removed and weighed. The piece of skin 1.5 cm. in diameter was cut into fine pieces and ground in a mortar. Physiological saline solution was added to make a dilution of 1–10. The suspension was centrifuged and the supernatant used for precipitin tests. This method of extraction is evidently ineffective because the concentration of globulin showed scant change as the interval after injection increased. It is noteworthy that tests of the animals' sera following injection of globulin into inflamed or normal cutis were negative.

The Penetration of Horse Serum Globulin into the Serum after Injection into Normal and Inflamed Subcutaneous Tissue.—5 cc. of a 1–10 dilution of 24 hour broth culture of *Staphylococcus aureus* were injected subcutaneously into the flanks of rabbits. On the following day a thick, indurated area measuring about 4 to 6 cm. in diameter was seen. 2 cc. of a 3 per cent globulin solution were injected into this area in one group of animals 17 hours and in another 25 hours after the beginning of inflammation. A similar injection was made into a normal animal at the same time. The animals were bled from the ear vein at intervals varying from 5 to 24 hours after the injections of the globulin, and precipitin tests were performed on the sera.

Table I shows the results of tests made to determine the concentration of globulin in the blood serum at different intervals after injection into inflamed and into normal subcutaneous tissue. The three graphs of Fig. 1 are constructed from these tables and show the maximum titers at which globulin was demonstrable in the serum. The graphs of subsequent figures have been similarly constructed but tabulation of the data is omitted.

Graph 1 in Fig. 1 shows that the concentration of protein in the serum after simultaneous injection of inflammatory irritant and protein is for 24 hours almost the same as that following injection of protein alone. Inflammation that has lasted 17 or 25 hours (graphs 2 and 3 of Fig. 1) delays the penetration of globulin into the blood so that the concentration of protein in the serum at corresponding time intervals after injection of the protein is less than that of animals which received subcutaneous injections of globulin alone. 24 hours after the injection of the protein there is still slightly more horse serum globulin in the serum of the control animals than in that of the animals with inflammation. However, at this time there has been opportunity for considerable absorption of the protein from the inflamed areas.

The Penetration of Horse Serum Globulin into Serum after Injection into Inflamed Peritoneal Cavities.—5 cc. of a 1–10 dilution of 24 hour broth culture of *Staphylococcus aureus* were injected into the peritoneal cavities of rabbits. 1 to 2 cc. of a 3 per cent solution of horse serum globulin were injected simultaneously or on the following day. The same amounts of protein were also injected into normal rabbits. The rabbits were bled from the ear vein at stated intervals after the injection and precipitin tests were performed on the sera.

TABLE I

Presence of Horse Serum Globulin in the Blood Stream at Different Intervals Following Its Injection at the Site of Inflammation in the Subcutaneous Tissue Compared with That Following Injection into Normal Tissue

Time after injection of globulin	Dilutions					Dilutions				
	1:1	1:3	1:9	1:27	1:81	1:1	1:3	1:9	1:27	1:81
	After simultaneous injection of inflammatory irritant and globulin					After injection of globulin in normal skin				
hrs.										
5	+	tr.	0	0	0	+	sl. tr.	0	0	0
5	+	sl. tr.	0	0	0	+	" "	0	0	0
7	+	" "	0	0	0	+	" "	0	0	0
7	++	+	0	0	0	++	+	tr.	0	0
11	–	+	–	0	0	++	+	0	0	0
24	++	+	0	0	0	++	++	tr.	0	0
24	+++	++	+	0	0	++	+++	++	tr.	0
	With inflammation lasting 17 hrs.					After injection of globulin in normal skin				
5	sl. tr.	0	0	0	0	+	tr.	0	0	0
5	tr.	0	0	0	0	tr.	0	0	0	0
7	"	sl. tr.	0	0	0	–	+	+	sl. tr.	0
7	"	0	0	0	0	–	++	tr.	" "	0
11	–	+	sl. tr.	0	0	–	++	"	" "	0
24	++	+	" "	0	0	+++	++	+	" "	0
24	–	+	tr.	0	0	–	++	+	tr.	0
	With inflammation lasting 25 hrs.					After injection of globulin in normal skin				
5	0	0	0	0	0	++	+	0	0	0
5	0	0	0	0	0	0	0	0	0	0
7	tr.	0	0	0	0	++	+	tr.	sl. tr.	0
7	0	0	0	0	0	+++	++	+	tr.	0
11	++	sl. tr.	0	0	0	+++	++	+	sl. tr.	0
11	+	tr.	0	0	0					
24	+++	++	tr.	sl. tr.	0	+	++	+	tr.	0
24	+++	+	sl. tr.	0	0					

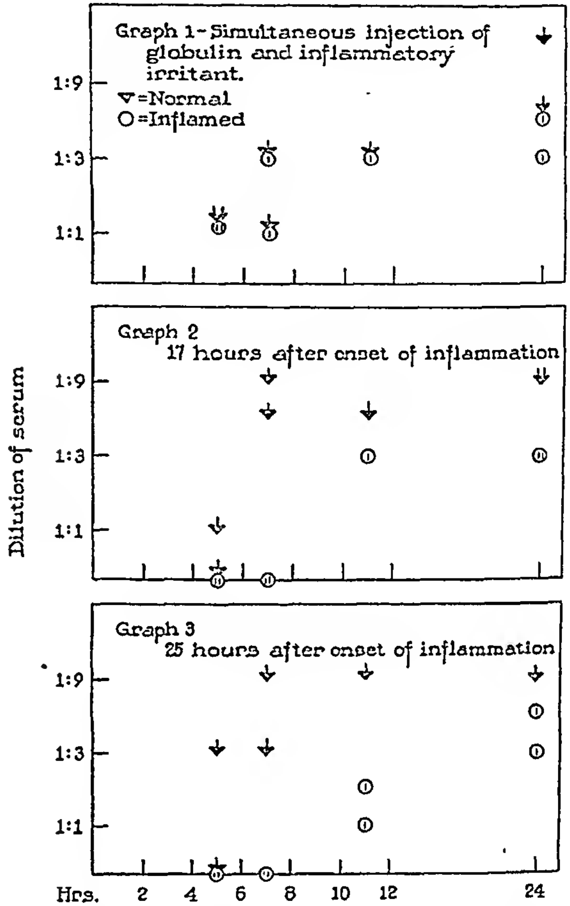

FIG. 1. The concentration of horse serum globulin in the circulating blood afte its injection into normal and inflamed areas. The triangles in this and subse quent figures show the titer of globulin in the blood serum of rabbits after it injection into normal subcutaneous tissue, and the circles show its concentratio after injection into inflamed subcutaneous tissue. The short lines above the tri angles or within the circles indicate the number of observations at a given point Graph 1 shows the concentration of horse serum globulin in the blood of rabbit after simultaneous injection of globulin and the inflammatory irritant compare with its concentration at corresponding intervals after injection of globulin alon Graph 2 shows the concentration of globulin in the blood serum of animals tha have received globulin 17 hours after the onset of inflammation compared with th corresponding controls. Graph 3 shows the concentration in the blood serum o rabbits that received globulin 24 hours after the onset of inflammation.

Fig. 2 shows that the penetration of horse serum globulin into the serum of the animals with the inflamed peritoneal cavities is delayed,

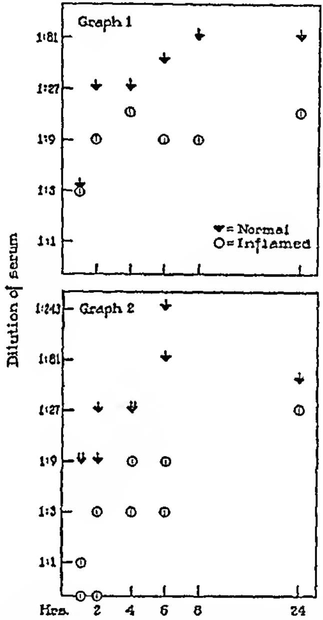

FIG. 2. The concentration of horse serum globulin in the circulating blood after its injection into normal and inflamed peritoneal cavities; graph 1, with simultaneous injection of globulin and inflammatory irritant, and graph 2, with injection of globulin 18 hours after onset of peritonitis, in both instances compared with controls.

even when the irritant is injected simultaneously with the protein (graph 1), but retardation of absorption is greater when the injection of globulin is made 18 hours after the onset of inflammation (graph 2).

The Penetration of Crystalline Egg Albumin into the Serum after Injection into Normal and Inflamed Subcutaneous Tissues.—Rabbits were injected subcutaneously with 3 cc. of staphylococcus vaccine or 2 cc. of a 1–10 dilution of a 24 hour broth culture of *Staphylococcus aureus*. On the following day, 1 or 2 cc. of 3 per cent egg albumin were injected into the subcutaneous tissue of normal animals. The rabbits were bled at intervals and precipitin tests were performed on the sera.

The results are given in Fig. 3. There is a slightly higher concentration of egg albumin in the serum of the normal animals than in the animals with inflamed subcutaneous tissue. This difference was most apparent 2 to 4 hours after injection. 24 hours after injection most of the egg albumin had left the sera of both groups of animals.

The Penetration of Crystalline Egg Albumin into the Serum after Injection into Normal and Inflamed Peritoneal Cavities.—Rabbits were injected intraperitoneally

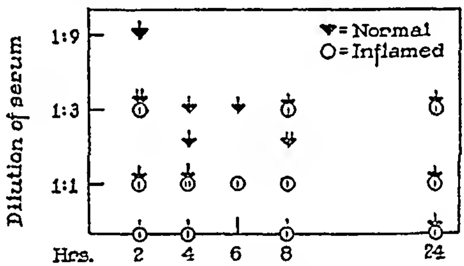

FIG. 3. The concentration of crystalline egg albumin in the circulating blood after its injection into the normal subcutaneous tissue and into the inflamed subcutaneous tissue from 19 to 27 hours after the onset of inflammation.

with 5 to 10 cc. of a 24 hour staphylococcus culture, diluted 1–10, or with 10 cc. of aleuronat starch mixture. Simultaneously, or on the following day, 2 to 8 cc. of 5 per cent egg albumin solution were injected intraperitoneally into these animals, and into normal rabbits. Blood was drawn at intervals and the sera were used for precipitin tests.

The results are given in Fig. 4. Following the simultaneous injection of inflammatory irritant and crystalline egg albumin the concentration of the latter in the blood serum differs little from that of animals that received albumin alone (graph 1). 2 hours after intraperitoneal administration of crystalline egg albumin from 18 to 22 hours after onset of inflammation (graph 2) there was more egg albumin in the sera than in those of the normal animals that received crystalline egg albumin. This difference was still apparent 4 to 6 hours

after injection. By 24 hours most of the egg albumin had disappeared from the sera of both groups of animals.

In a few experiments urine from the injected animals was tested for the presence of egg albumin. It was found that the protein could be detected in the urine as early as 2 hours after injection. The amount of protein excreted seemed to have no constant relationship to the amount in the circulating blood.

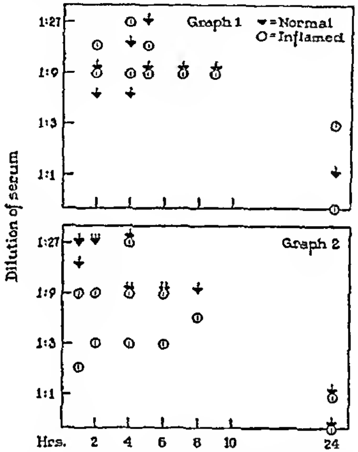

FIG. 4. The concentration of crystalline egg albumin in the circulating blood after its injection into the normal and inflamed peritoneal cavity; graph 1, with simultaneous injection of crystalline egg albumin and inflammatory irritant, and graph 2, with injection of globulin from 18 to 22 hours after onset of peritonitis, in both instances compared with controls.

The Influence of Inflammation on the Absorption of Carbohydrates

The pneumococcus Type I specific polysaccharide[1] and glucose were used in these experiments. The polysaccharide had been prepared according to the method of Heidelberger, Kendall and Scherp (16).

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[1] The polysaccharide was obtained through the courtesy of Dr. Kenneth Goodner and Dr. Frank Horsfall of The Rockefeller Institute for Medical Research.

Earlier information (Heidelberger, 17) has indicated that the pneumococcus type specific carbohydrates are diffusible compounds of low molecular weight. However, the polysaccharides prepared by the newer methods, which ensure against their hydrolysis, appear to be more indiffusible compounds. Thus, the recent work of Avery and Goebel (18) demonstrates that 7 days after the intravenous injection of 17 mg. of pneumococcus Type I polysaccharide into rabbits, this substance may still be detected in the circulating blood. Babers and Goebel (19) have studied the rate of diffusion of the pneumococcus Type III polysaccharide and have concluded that it is an extremely indiffusible compound, the molecular weight of which they calculate to be 118,000.

Antisera against the polysaccharide were prepared by the repeated intravenous injection of a Type I pneumococcus vaccine into rabbits. A smooth organism which had recently been passed through mice was used in the preparation of the vaccine. The titer obtained was between 1–1,000,000 and 1–5,000,000.

A 0.1 per cent solution of the carbohydrate was prepared by dilution with physiological saline solution. It was sterilized by heating for 1 hour at 56°C. Preliminary experiments were made to determine the diffusibility of the compound in the animal body. In the preliminary experiments, 2.5 mg. and 5.0 mg. respectively of the Type I carbohydrate were injected intravenously into rabbits. The animals were bled from the ear vein and the sera used for precipitin tests.

It was found that the specific polysaccharide titer of the blood dropped somewhat in the first 10 minutes after intravenous injection. Thereafter the titer remained approximately constant for 24 hours, and small amounts of the substance were still present in the circulating blood 72 hours after injection. Very small amounts of the polysaccharide appeared in the urine during the first day after injection.

The Penetration of Pneumococcus Type I Polysaccharide into the Serum after Injection into Normal and Inflamed Peritoneal Cavities.—5 mg. of pneumococcus Type I polysaccharide in 5 cc. of physiological saline solution were injected intraperitoneally into normal rabbits and into those which had received 5 cc. of a 1–10 dilution of staphylococcus culture intraperitoneally on the previous day.

The results are given in Fig. 5. There is a consistent difference between the penetration of the pneumococcus Type I polysaccharide into the serum from normal and into that from inflamed peritoneal cavities. 2 hours after injection more carbohydrate is present in the blood of the normal than of the inflamed animal. This difference is still apparent although to a lesser extent 24 hours after injection.

The Disappearance of Glucose after Injection into Normal and Inflamed Peritoneal Cavities.—5 cc. of *Staphylococcus aureus* vaccine were injected intraperitoneally into rabbits. On the following day 10 to 100 cc. of 5 per cent glucose solution were injected intraperitoneally into the treated rabbits and into normal animals. The animals were killed by air injection 30 minutes to 60 minutes after the injection of the glucose. In the rabbits that received 10 to 20 cc. of glucose, 100 cc. of saline solution were introduced into the peritoneal cavity immediately after death. The abdominal cavity was then opened and the contents allowed to drain into a large funnel. An additional 100 cc. of saline were used to wash out the peritoneal cavity. When 100 cc. of glucose had been injected, the abdominal cavity was opened immediately after death, and the contents allowed to drain into a funnel. The remaining procedure was the same. Glucose was determined by the method of Miller and Van Slyke (20).

Table II shows that the absorption of glucose from the inflamed peritoneal cavity proceeds as rapidly as absorption from the normal

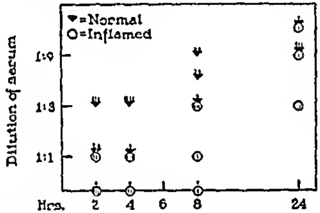

FIG. 5. The concentration of the specific polysaccharide of pneumococcus Type I in the circulating blood after its injection into the normal peritoneal cavity and into the inflamed peritoneal cavity from 13 to 20 hours after the onset of peritonitis.

peritoneal cavity. When large amounts of glucose are injected (5100 mg.), there may be more rapid absorption from the inflamed than from the normal peritoneal cavity.

The Influence of Inflammation on the Absorption of Dyes

The Disappearance of an Indiffusible Dye from the Inflamed Cutis.—A 1 per cent solution in 0.5 per cent saline of trypan blue was used. 0.05 to 0.1 cc. of the solution was injected into the skin of rabbits into which 0.2 cc. of a 24 hour broth culture of *Staphylococcus aureus* had previously been injected. The sites chosen were the anterior surface of the tip of the ear or the abdominal wall. The contralateral normal side was used as a control. The spread of the dye was observed at varying intervals and recorded by means of colored drawings.

Trypan blue (Table III) remained localized within a small area of the inflamed skin, whereas a wide diffuse coloration appeared in the

TABLE II

Absorption of Glucose after Injection into the Peritoneal Cavity

Experiment No.	Weight	Amount injected	Time glucose remained in body	Amount recovered	Absorbed
	gm.	*mg.*	*min.*	*mg.*	*per cent*
	Animals with Inflamed Peritoneal Cavities				
1	1835	525	32	113.3	78.5
2	1700	1000	33	365.0	63.5
3	1950	1000	35	474.0	52.6
4	1680	1000	30	698.0	30.2
5	1650	5100	60	2005.0	60.6
6	1690	5100	60	1985.0	61.0
	Animals with Normal Peritoneal Cavities				
1	1915	525	32	203.9	61.1
2*	1725	1000	40	335.0	66.5
3	1750	1000	35	420.0	58.0
5	1630	5100	60	2550.0	48.2
6	1680	5100	60	2475.0	51.4

* Cysticerci of *Taenia pisiformis* were present in the abdominal cavity and upon the omentum and liver.

TABLE III

Spread of Trypan Blue Injected into Normal and Inflamed Intracutaneous Sites

Experiment No.	Interval after injection of dye	Colored area in normal site	Colored area in inflamed site	Difference in color	Comment
	hrs.	*sq. mm.*	*sq. mm.*		
1	8	300	28	Inflamed darker	
2	24	392	32	" "	2 days later inflamed site is colorless, normal measures 306 sq. mm.
3	24	150	48	—	2 days later inflamed site measures 12 sq. mm., normal 338 sq. mm.
4	24	450	90	—	2 days later inflamed site measures 48 sq. mm., normal 576 sq. mm.

normal skin. Nevertheless, the ultimate disappearance of the dye was sometimes effected more rapidly from the inflamed than from the normal skin. This occurred several days after injection.

The Disappearance of Brom Phenol Blue from the Inflamed Cutis.—0.2 to 0.5 cc. of staphylococcus culture was used to produce the inflammatory reaction in the skin of rabbits. The sites chosen were the anterior abdominal wall about 4 cm. from the midline, and the skin of the anterior surface of the tip of the ear. 18 to 24 hours after the injection of the irritant, 0.05 or 0.1 cc. of brom phenol blue (0.2 to 0.4 per cent solution in physiological saline) was injected into the inflamed site

TABLE IV

Disappearance of Brom Phenol Blue after Injection into Normal and Inflamed Cutaneous Areas

Experiment No.	Amount of dye injected	Time from injection of dye to last observation	Colored area remaining in		Color difference
			Inflamed site	Normal site	
		hrs.	*sq. mm.*	*sq. mm.*	
1	0.05 cc. 1–10	3	9	42	Normal site lighter
2	0.05 " 1–10	3	2	16	None
3	0.05 " 1–10	3	14*	80	"
4	0.05 " 1–10	3	4	156	"
5	0.05 " 1–20	3	0	6	—
6	0.05 " 1–40	2	0*	20	—
7	0.05 " 1–40	2	0	16	—
8	0.05 " 1–10	7	80	70	Inflamed site much lighter
9	0.05 " 1–10	7	72	120	Inflamed site lighter
10	0.05 " 1–10	7	64	143	Normal site lighter
11	0.05 " 1–10	7	48	90	None
12	0.05 " 1–10	4	56	30	Inflamed site lighter
13	0.05 " 1–10	7	12	42	" " "
14	0.05 " 1–20	7	2†	0	—
15	0.05 " 1–20	7	1†	0	—
16	0.05 " 1–20	7	6†	0	—
17	0.1 " 1–120	24	4†	0	—
18	0.1 " 1–20	24	1†	0	—

* 48 hour inflammation.

† Necrosis.

and into the contralateral normal side. After the wheal caused by the injection had disappeared, the dye-stained areas were measured and recorded by means of colored drawings. Further drawings were made at intervals until the dye had disappeared in one or both of the injected sites. When the observations were not continued until the disappearance of the dye, the last reading is given.

The results are listed in Table IV. This table shows that inflammation facilitates the disappearance of this dye if the inflammatory

process does not proceed to necrosis. When necrosis appears the dye becomes firmly fixed in the necrotic tissue.

The Disappearance of Phenol Red from the Inflamed Cutis.—Sterile ampoules of phenol red, containing 6 mg. per cc.,[2] were used. 0.2 to 0.3 cc. of the dye was injected intracutaneously into normal areas and into those which had previously been treated by the injection of 0.1 to 0.3 cc. of *Staphylococcus aureus* cultures. At stated intervals the animals were killed and the injected areas, measuring 1.5 cm. in diameter, were removed. The tissues were weighed and cut into fine strips. They were then ground in a mortar and repeatedly extracted with 95 per cent

TABLE V

Extraction of Phenol Red from Normal and Inflamed Cutaneous Areas

Experiment No.	Duration of inflammation before injection of dye		Time dye remained in body	Amount injected	Amount recovered from	
					Inflamed site	Normal site
	hrs.	*min.*	*min.*	*mg.*	*mg.*	*mg.*
1	1	00	15	1.2	0.18	0.38
2	1	00	15	1.2	0.28	0.35
3	1	00	30	1.2	0.08	0.30
4	3	00	30	1.2	0.28	0.19
5	3	00	30	1.2	0.22	0.11
6	22	15	30	1.8	1.5*	0.44
7	22	00	40	1.2	0.23	0.24
8	22	00	40	1.2	0.11	0.30
9	17	15	45	1.2	0.22	
10	17	15	45	1.2	0.19	0.098
11	3	00	50	1.8	0.52	0.39
12	18	00	50	1.8	0.60	0.41

* Marked necrosis.

alcohol, until the extracts showed no further pink color on the addition of alkali. Colorimetric determinations of the phenol red content of the extracted fluid were made. This method had previously been found satisfactory by Marshall and Vickers (21).

The results are given in Table V. Great variations occur in the disappearance of phenol red from the inflamed cutaneous areas. In the first 15 minutes after injection the dye appears to be absorbed more rapidly from the inflamed site. In the later stages of absorption no difference can be found between the disappearance of the dye from

[2] Prepared by Hynson, Westcott and Dunning.

the normal and from the inflamed areas. However, in examining these results the familiar observation that phenol red readily combines with plasma proteins must be taken into consideration. It was found by Marshall and Vickers (21) that in the rabbit 90 to 98 per cent of phenol red may be bound to plasma proteins. The effect of the combination would not be apparent when the dye is present in excess; but when inflammation is far advanced or only a small amount of dye is present, as in the late stages of absorption, there is presumably an excess of protein over dye and combination between them probably occurs.

The Excretion of Phenol Red in the Urine after Injection into Normal and Inflamed Subcutaneous Tissue.—1 cc. of phenol red was injected into normal and inflamed subcutaneous tissues of male rabbits weighing 1800 to 2300 gm. Inflammation was produced by the injection of 3 cc. of a 1–10 dilution of a 24 hour staphylococcus culture or 5 cc. of an aleuronat starch mixture. The animals were given 100 cc. of water by stomach tube about 1 hour before the injection of the dye. Another 50 cc. of water was given 1 to 2 hours later. Urine was obtained by catheterization. In some instances numerous difficulties were experienced in obtaining suitable specimens; it was found that animals developed anuria for several hours after the catheterization was attempted and it was necessary to wait until a free flow of urine occurred before starting the experiment. After each catheterization the bladder was washed with 10 cc. of water to ensure complete collection of the urine. Quantitative colorimetric estimations of the amount of phenol red were made according to the method of Rowntree and Geraghty (22).

The results of the experiments are given in Table VI which shows the average percentage excretion of the dye at a given time as well as the total excretion. The results in the two groups of animals are practically identical. They indicate that there is rapid excretion of phenol red after its injection into both normal and inflamed subcutaneous tissues.

The Excretion of Phenol Red in the Urine after Injection into Normal and Inflamed Peritoneal Cavities.—The procedures were identical with those given above, except that the dye was injected into the peritoneal cavities instead of the subcutaneous tissues. Inflammation was produced by the injection of 5 cc. of a 1–10 dilution of staphylococcus culture or 10 cc. of aleuronat starch mixture. Phenol red (6 mg.) was introduced into the peritoneal cavity from 18 to 24 hours after the injection of the irritant.

TABLE VI

Percentage Excretion of Phenol Red in Urine after Subcutaneous Injection of 6 Mg.

Experiment No.	Time (hours) ½	1	2	3	4	Total
	per cent	per cent	per cent	per cent	per cent	per cent
	Animals with Inflamed Subcutaneous Tissues					
1	17.5	25.0	15.0	12.5	1.7	71.7
2	12.6	20.0	24.2	12.0	5.9	74.7
3	26.8	26.6	18.5	5.0	0.6	77.5
4	11.1	22.9	29.4	14.2	5.0	82.6
5	25.0	25.5	14.1	—	5.6	70.5
Average...	18.6	24.0	20.3	10.9	3.7	77.6
	Animals with Normal Subcutaneous Tissues					
1	25.0	22.5	12.3	5.0	1.5	66.5
2	20.0	21.0	29.7	3.6	5.6	79.9
3	14.1	25.0	23.8	8.3	7.1	78.3
4	14.1	36.7	8.4	14.9	6.1	80.2
Average...	18.3	26.3	18.6	8.0	5.0	76.2

TABLE VII

Percentage Excretion of Phenol Red in Urine after Intraperitoneal Injection

Experiment No.	Time (hours) ½	1	2	4	Total
	per cent	per cent	per cent	per cent	per cent
	Animals with Inflamed Peritoneal Cavities				
1	15.7	24.5	20.0	17.6	76.8
2	22.7	19.5	18.1	9.3	69.6
3	17.0	20.8	23.0	7.3	68.1
4	23.3	18.8	9.9	2.6	54.6
Average....	19.7	22.15	17.8	9.2	68.8
	Animals with Normal Peritoneal Cavities				
1	21.4	28.6	15.6	13.0	78.6
2	21.0	26.8	16.6	7.2	71.6
3	9.5	23.5	14.4	11.3	58.7
4	16.6	24.3	15.6	5.6	62.1
Average....	17.1	25.8	15.5	9.2	67.6

The results are given in Table VII. No differences are seen between absorption of the dye from normal and inflamed peritoneal cavities, as indicated by the rate of its excretion in the urine.

The Disappearance of Phenol Red after Injection into Normal and Inflamed Subcutaneous Tissues.—5 cc. of a 1–10 dilution of a 24 hour broth culture of *Staphylococcus aureus* or 5 cc. of aleuronat starch mixture were injected subcutaneously into the dorsal surface of the thighs of rabbits. On the following day

TABLE VIII

Phenol Red Recovered from the Site of Inflammation after Subcutaneous Injection of 6 Mg.

Experiment No.	Duration of inflammation before injection of dye		Time dye remained in body	From inflamed site	From normal site
	hrs.	*min.*	*min.*	*mg.*	*mg.*
	0	00	Excised immediately		5.58
	0	00	" "		3.3 (3.6 injected)
1	25	45	10	4.8	5.2
2	24	00	15	3.6	4.0
3	24	00	15	3.6	3.6
4	14	00	16	3.2	3.4
5	16	00	20	1.6	2.1
6	24	45	25	3.0*	2.2
7	1	00	25	1.2	2.4
8	21	00	30	1.8	2.4
9	24	20	35	1.3	1.6
10	21	30	40	0.88	2.5
11	22	20	50	0.93	1.7
12	25	00	110	0.55	0.35

* Abscess formation.

1 cc. (6 mg.) of phenol red was injected into the inflamed area and into the contralateral normal area. At stated intervals the rabbits were killed by air injection and a circular piece of skin measuring 4 cm. in diameter, the center of which corresponded with the site of injection, was removed. The underlying fascia containing dye was also excised. Extraction with 95 per cent alcohol was performed by the procedure used after intracutaneous injection. The amount of dye extracted was determined colorimetrically.

The results are shown in Table VIII. In all but two of the experiments the phenol red has disappeared more rapidly from the inflamed

than from normal subcutaneous tissue. In one instance an abscess had formed and in the other about 2 hours had elapsed between injection of the dye and its extraction.

DISCUSSION

The substances used in these experiments fall into the general categories of proteins, carbohydrates and dyes. Of the proteins, serum globulin is a very slowly diffusible compound with a molecular weight of approximately 140,000 (see Cohn, 23). Egg albumin is composed of smaller, more diffusible molecules weighing 34,500 (Svedberg, 24).

Several investigators (2, 3), have demonstrated that the parenteral absorption of proteins is accomplished chiefly by the lymphatic system. Nevertheless, a certain portion of injected protein may enter the blood vessels directly (3). This portion is probably larger in the case of the more diffusible egg albumin than in the case of globulin. In our experiments differences in diffusibility of the two proteins are indicated by the fact that egg albumin reaches a maximum concentration in the blood about 2 to 4 hours after intraperitoneal injection. Thereafter, the concentration in the blood falls sharply, and large amounts of the albumin appear in the urine. On the contrary, the maximum concentration of globulin in the blood is attained only 24 hours after intraperitoneal injection, and the excretion of this protein is very slow.

When crystalline egg albumin or horse serum globulin is injected into inflamed subcutaneous areas or inflamed peritoneal cavities, its absorption is definitely retarded. This retardation is more conspicuous in the case of globulin than of egg albumin.

A further difference in the behavior of these proteins is seen when they are injected simultaneously with the irritant. Under these circumstances, concentration of egg albumin in the serum of the animal with the inflamed peritoneal cavity is higher than the concentration in the serum of the normal animal. The difference is apparent 2 hours after the injection. This observation appears to parallel the experiments of Okuneff (25) who found that absorption of trypan blue from the subcutaneous tissue is accelerated when this dye is injected simultaneously with an irritant. Hoehne (7) has shown that the

absorption of bacteria is similarly accelerated in the early stages of inflammation.

Data showing the effect of inflammation on the absorption of two carbohydrates have been given. On the one hand, the pneumococcus Type I specific polysaccharide is prevented from leaving the inflamed peritoneal cavity. On the other, the absorption of glucose is unaffected or accelerated by inflammation. These compounds differ sharply in diffusibility. The pneumococcus polysaccharide is an exceedingly indiffusible substance, as demonstrated by the difficulty with which it leaves the blood after intravenous injection. It is likely that, in common with other indiffusible substances, it is absorbed chiefly by way of the lymphatic system (3).

The extreme diffusibility of glucose is a familiar property of this substance. It is absorbed readily after parenteral injection and undoubtedly enters the blood vessels directly. Inflammation in no way deters the absorption of this carbohydrate. The difference in the rate of absorption of the two carbohydrates in the presence of inflammation is apparently related to differences in their diffusibility.

Similarly, the effect of inflammation on the absorption of dyes depends largely on the diffusibility of the compounds. Phenol red (phenolsulfonphthalein) is the most diffusible of the dyes studied. The excretion of this dye in the urine after injection into subcutaneous tissue or the peritoneal cavity indicates that there is no difference between its absorption from normal and from inflamed areas. However, direct extraction of phenol red from the tissues clearly demonstrates an acceleration in the absorption of this dye from inflamed subcutaneous areas. The method of direct extraction is undoubtedly a more accurate index of absorption than is the estimation of excretion. The latter is subject to several experimental errors, such as those that occur in collection of urine samples. It introduces also the factor of renal efficiency, which differs widely in different animals.

Phenol red has the property of entering a chemical or physical union with plasma proteins when these are present in excess (Marshall and Vickers, 21). Because of this property, the diffusible dye may readily be converted into a relatively non-diffusible compound. Furthermore, the ratio of combined to uncombined dye will vary with the amount of protein present, the pH of the solution, etc. These

considerations make it difficult to estimate differences in the absorption of unaltered phenol red from normal and inflamed cutis, where the amount of dye injected is small and protein may be present in excess.

The diffusible dye brom phenol blue is absorbed more rapidly from inflamed than from normal skin. However, when necrosis occurs in the inflamed area, it inhibits the absorption of the dye. Where there is necrosis, inflammation, which can occur only in living tissues, ceases. Thrombosis of blood and lymphatic vessels and the formation of a fibrous wall around the necrotic area sharply delimit the dead from the living tissue. Conspicuous inhibition of absorption was noted also in several experiments in which phenol red was injected into necrotic tissue.

Trypan blue shows little tendency to spread in the inflamed skin in contrast to the wide, diffuse coloration which appears when the dye is injected into normal skin. Even though the dye ultimately disappears from the inflamed site more rapidly than from the normal one, it remains localized in a relatively small area throughout the period of disappearance.

The adsorptive properties of many dyes raise some objection to their use. Okuneff (25) has shown that charcoal, bolus alba and proteins adsorb trypan blue: the dye is partially decolorized. Despite this fact he concluded (11), as had Menkin, that inflammation definitely retards the absorption of this dye. In the present experiments the fact that the trypan blue remained darker in color in the inflamed than in the normal site argues against any considerable adsorption by the protein in the inflamed tissue.

In general, these experiments indicate that inflammation inhibits the absorption of slowly diffusible compounds and accelerates the absorption of readily diffusible ones.

The acceleration of absorption of diffusible compounds in the inflamed area is undoubtedly related to the rapid flow of blood since these compounds enter the blood vessels directly. The mechanism of the inhibition of absorption of the relatively indiffusible materials is less clear. The process may be one that concerns the lymphatics, for the substances affected by it depend largely on the

has suggested that the presence of fibrin in the tissue spaces and lymphatic vessels prevents the entrance of substance into the latter.

The agents that produce inflammation are commonly of a bacterial or parasitic nature. A large body of experimental data (6–9) leaves no doubt that bacteria are inhibited or prevented from leaving the inflamed area, within which the microorganisms tend to be dissolved or destroyed. During this process, the fate of toxic bacterial products is of the utmost importance, for these may offer greater potential dangers than the bacteria themselves. Toxins and other primary bacterial derivatives are soluble, whereas the bacterial body is particulate; they may readily gain access to vital organs by way of the blood. Localization of these products in the inflamed area, which is well prepared to destroy and digest them, may prevent widespread bodily injury.

The inhibiting mechanism evidently does not operate with equal facility upon all substances. The absorption of globulin from the inflamed area is retarded more readily than the absorption of egg albumin, but complete inhibition of the absorption of soluble substances has not been observed. On the contrary, several investigators have reported that inflammation may completely prevent the entrance of bacteria into the blood. Thus, Opie (9) found that the penetration of streptococci from the inflamed peritoneal cavity into the blood might be completely prevented. Nevertheless, some of the animals in which sterile blood cultures were obtained for hours after injection of the bacteria, showed symptoms of toxemia before a terminal bacteremia was observed.

It appears probable that the degree of inhibition of absorption from the inflamed area is related to the diffusibility of the compound studied; greater indiffusibility is accompanied by greater inhibition of absorption. Bacteria are localized more effectively than their derivatives, among which there are probably further differences according to diffusibility.

In order that the inflammatory process may pursue a favorable course it is important that both the injurious and the defensive substances be retained within it. Mobilization of such substances in the inflamed site is well recognized. "The inflammatory reaction affords means by which various substances, notably enzymes, are

delivered in unusual quantity in response to unusual local need" (Opie, 26). It is notable that antibodies and many known enzymes are proteins and therefore relatively indiffusible compounds. They, together with indiffusible bacterial and tissue products, are retained in the inflamed site where interaction between them occurs.

On the contrary, it is important that end products of enzymatic digestion and other diffusible metabolites produced in the inflamed site should not accumulate there. Such accumulation would tend to add further injury to an already injured tissue. Our results indicate that rapid absorption of these substances from the inflamed site occurs: upon entrance to the blood they are readily excreted.

CONCLUSIONS

1. Inflammation retards the absorption of horse serum globulin and crystalline egg albumin from the peritoneal cavity and subcutaneous tissue, but retardation of the absorption of crystalline egg albumin is less than that of globulin, which is less diffusible.

2. Inflammation retards the absorption of the specific polysaccharide of pneumococcus Type I from the peritoneal cavity; inflammation may accelerate, but does not hinder, the absorption of glucose from the peritoneal cavity.

3. Inflammation retards the spread of trypan blue in the skin, but accelerates absorption from the skin of the more diffusible dye, brom phenol blue.

4. Phenol red is excreted in the urine with equal rapidity after injection into normal and into inflamed subcutaneous tissue or into normal and into inflamed peritoneal cavities. Direct extractions of phenol red from inflamed subcutaneous sites indicate that inflammation accelerates the absorption of the dye from these areas.

5. Inflammation retards the absorption of the indiffusible proteins, carbohydrates and dyes; it tends to accelerate the absorption of the diffusible carbohydrates and dyes.

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A STABLE HEMOLYSIN-LEUCOCIDIN AND ITS CRYSTALLINE DERIVATIVE ISOLATED FROM BETA HEMOLYTIC STREPTOCOCCI*

By E. J. CZARNETZKY, Ph.D., ISABEL M. MORGAN, and STUART MUDD, M.D.

(*From the Department of Bacteriology, School of Medicine, University of Pennsylvania, Philadelphia*)

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The hemolysis produced by various strains of streptococci is a characteristic of such importance that it serves as a basis for classification. Of the large amount of work on the hemolysins of the true or β hemolytic streptococci, the greater part has been done with filtrates of broth cultures. Thus the active hemolysin was contained in a mixture composed of the known constituents of the broth, and the unknown products of cellular metabolism, and products of their interactions. In general the hemolysins have fallen into two classes, those which are stable, and those which are labile to oxygen. The hemolysin to be reported here is a pure substance, free of the extraneous constituents of the culture fluid. This hemolysin is stable to oxygen, as well as to heat and alterations in pH within the physiological range. It will be referred to as the stable hemolysin (S. H.).

Among the earlier reports on oxygen-labile hemolysins may be mentioned that of McLeod (1) who in 1912 reported a hemolysin from streptococcal filtrates which was labile to heat as well as to oxygen. Neill and Mallory (2) in 1926 reported a reversibly oxidizable streptolysin. Later Todd (3) demonstrated that hemolysins which were oxygen-labile, antigenic and neutralizable by immune sera occurred in streptococci of Lancefield's group A only. Oxygen-stable hemolysins on the other hand were not confined to group A strains but were produced by strains of animal as well as of human origin, falling into groups A, B, C, D, and E. The oxygen-stable hemolysin of Todd was not neutralizable by antistreptolysin and appeared to be non-antigenic.

Since the earliest recognition of bacterial leucocidins, many fragmentary reports

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have appeared. As with the hemolysins, there is an apparent lack of parallelism between the various leucocidins reported, perhaps due to the fact that they were present in a complex mixture of broth culture filtrate. Comparisons of such properties as heat and oxygen lability are of doubtful significance. Much of the discussion centers around the identity of the hemolysin and leucocidin of a given strain of streptococci.

Weld (4) showed that under certain conditions serum extracts of hemolytic streptococci are markedly hemolytic and are toxic for mice when injected intravenously, causing hemoglobinuria, anemia and death. Further studies showed that the principal pathologic finding in mice was marked degeneration of the renal tubular epithelium.

Channon and McLeod (5) reported a thermolabile toxin from serum broth cultures of hemolytic streptococci. This toxin showed a marked lytic activity on leucocytes, and they believed it to be the same as the hemolysin.

Nakayama (6) tested for leucocidin from streptococcal broth filtrates by two methods: (*a*) by the loss of ameboid motion of leucocytes when mixed with leucocidin, and (*b*) by the bioscopic method of Neisser and Wechsberg (7); that is, the effect of the leucocidin in inhibiting the reduction of methylene blue by leucocytes. A modification of the latter method has been used in the experiments reported here.

An extensive review by Evans (8) brought evidence for the non-identity of the streptococcal leucocidin (heat-stable) and the hemotoxin (heat-labile) which were obtained from hemolytic streptococcal broth culture filtrates. Gay and Oram (9) largely confirmed the work of Evans and described the action of streptococcal leucocidin on cells other than the polymorphonuclear neutrophils.

The stable hemolysin-leucocidin (S. H.) reported here was obtained in pure, that is, homomolecular form. Thus the activity of the S. H. was due to a single species of molecule acting as hemolysin and as leucocidin. The parallel activity of S. H. as hemolysin and as leucocidin will be brought out, dealing with the material in its "native" form and in the crystalline derivative of this. The crystalline derivative will be referred to as C. S. H.

Description of the Strains of Organisms Used

Streptococcus hemolyticus. Lancefield group A.

Strain 1896. Falls into none of Griffith's original types. Original source lobar pneumonia. Colony form mucoid.

Strain 1685. Griffith type 1. Original source erysipelas. Colony form mucoid.

Strain 1685G. Smooth (glossy) variant of the above. Injection of 0.1 ml. of culture not fatal to mice.

Strain S43G.

S. hemolyticus. Lancefield group B, strain O90R.
S. hemolyticus. Lancefield group C, strain F132.
S. hemolyticus. Lancefield group G, strain H46C.

Strains Used for Purposes of Control.—

S. viridans. Strain P25. Laboratory strain.
Staphylococcus albus. Colony form rough.
Bacterium typhosum. Strain O901. Old laboratory strain described by Felix.
S. pneumoniae. Type I. Colony form mucoid.

Fuller description of the strains of streptococci used will be found in a preceding paper (10).

Preparation of the Stable Hemolysin

The stable hemolysin can be prepared either (*a*) from the lyophile-dried whole bacteria, or (*b*) from lyophile-dried plate washings as starting material. The yield of S. H. in either case is less than 1 per cent. (*a*) The organisms are grown in neopeptone broth in sufficient amount to yield about 1 gm. of bacteria after drying by the lyophile pro[illegible] plates are centrifuged and [illegible] candle. The clear solutio[illegible] of the dried material is used for the preparation of the S. H. fraction.

The dried microorganisms or plate washings are extracted in a Soxhlet apparatus for 2 days with moist ethyl ether (5 parts water + 95 parts absolute ether; *i.e.*, a two-phase system, so that in the collecting flask a layer of water underlies the ether which refluxes over the dried organisms). This crude two-phase extract is then placed in a boiling water bath until all traces of the ether have been removed. From the remaining watery extract the stable hemolysin may be readily obtained by adding absolute acetone (about 8 volumes) until a brown precipitate of rubbery consistency comes down. The acetone is decanted, the precipitate dried and dissolved in physiological saline solution. This solution contains the S. H. in crude form.

When a purified preparation is desired, the watery extract obtained as described above is added to 1 liter of saline and centrifuged to remove extraneous matter. The supernatant fluid is distilled to dryness *in vacuo* over a boiling water bath, and taken up in 50 ml. of 50 per cent acetone, centrifuged, and the supernatant precipitated with 15 volumes of dry ethyl ether followed by 5 volumes of absolute acetone. The precipitate is removed and dried at 100°C. until all odor of acetone has disappeared. Further purification may be effected by repetition of the above process. All samples used for chemical analysis were carried through the purification process until they gave a constant nitrogen-phosphorus ratio on analysis. This required 6 to 8 purifications. Evidence for the purity of the samples studied will be brought out later in this paper.

Crystalline Derivative of the Stable Hemolysin

A crystalline derivative of the stable hemolysin (C. S. H.) was obtained by the following procedure.

100 mg. of purified S. H. of known homomolecularity were dissolved in 25 ml. of distilled water and alkalified with 1 ml. of 40 per cent NaOH. The mixture was heated on a boiling water bath for 10 minutes, and then cooled rapidly under the tap. On standing in the refrigerator overnight, in a tightly sealed flask, small peg-shaped crystals separated. They were recovered by centrifugation, and dissolved in a minimal amount of hot 95 per cent alcohol. On cooling, similar crystals separated. After three additional alcohol and water recrystallizations, the material was found to consist of a trisodium salt, a derivative of the S. H.

Serological Specificity of the Stable Hemolysin

Injections of 0.01 ml. of a 1:1000 dilution of S. H. three times per week for 4 weeks failed to produce precipitins against the S. H. fraction. S. H. from both cells and the plate washings were prepared from strains 1685 and 1896 and each preparation was injected into three rabbits.

However, the S. H. is precipitable by antisera prepared by the injection of whole organisms, and is therefore a hapten. The specificity of the S. H. is shown in Table I. The results show that the S. H. is species-specific, in that any of the S. H. preparations are precipitated by any of the sera against β hemolytic streptococci, but not by antisera prepared against hemolytic staphylococci, pneumococcus Type I, *B. typhosum* or the broth used in the growing of the organisms. Moist ether extracts of all the strains of β hemolytic streptococci tested yield S. H. with the above properties, whereas moist ether extracts of *S. viridans*, hemolytic staphylococci, *B. typhosum* and pneumococcus Type I, do not have similar chemical properties, are not active hemolytically, nor are they precipitated by any of the antisera against these organisms or against β hemolytic streptococci.

Thus, the lack of antigenicity of S. H., its species specificity and its presence only in β hemolytic streptococci indicate its similarity to Todd's (3) oxy-stable hemolysin.

The C. S. H. is not serologically active; it neither precipitates antisera prepared against streptococci, nor inhibits the precipitation of S. H. by such antisera.

TABLE I

The Specificity of the Stable Hemolysin as Determined by Precipitation Tests

Group	Type	Rabbit antiserum *versus*	Stable hemolysin derived from							
			1896 cells	1896 washings	1685 cells	1685 washings	1685G cells	O90R cells	F132 cells	H46C cells
A	1	1685	0344	0333	0333	0333	0333	0333	0333	0333
A	1	1685G	0344	0333	0331	0333	0333	0333	0333	0333
A	1	1685G*	0334	0333	0333	0333	0333	0333	0333	0333
A	6	1048	0344	0333	0333	0333	0333	0333	0333	0333
A	14	1850	0344	0333	0332	0333	0333	0333	0333	0333
A	18	2084	0344	0333	0333	0333	0333	0333	0333	0333
A	—	2169	0344	0333	0333	0333	0333	0333	0333	0333
A	—	1793	0344	0333	0333	0333	0333	0333	0333	0333
A	—	1896	0344	0333	0333	0333	0333	0333	0333	0333
B	—	O90R	0333	0333	0333	0333	0333	0333	0333	0333
C	—	F132	0111	0111	0111	0111	0111	0111	0221	0222
D	—	H69	0333	0333	0333	0333	0333	0333	0333	0333
F	—	H60R	0333	0333	0333	0333	0333	0333	0222	0333
G	—	H46C	0333	0333	0333	0333	0333	0333	0333	0333
H	—	F90A	0333	0333	0333	0333	0333	0333	0333	0333
		Stable hemolysin	0000	0000	0000	0000	0000	0000	0000	0000
		Staphylococcus	0000	0000	0000	0000	0000	0000	0000	0000
		Pneumococcus	0000	0000	0000	0000	0000	0000	0000	0000
		B. typhosum	0000	0000	0000	0000	0000	0000	0000	0000
		Broth	0000	0000	0000	0000	0000	0000	0000	0000
		Normal horse serum	0000	0000	0000	0000	0000	0000	0000	0000
		Normal rabbit serum	0000	0000	0000	0000	0000	0000	0000	0000

The four figures under each antigen indicate the amount of precipitate in the saline control, and the antigen diluted 10^{-3}, 10^{-4} and 10^{-5}, respectively.

All antisera, with the exception of 1685G*, were prepared by the injection of living organisms. 1685G* antiserum was prepared by the injection of heat-killed organisms.

The precipitation tests were carried out with 0.5 ml. volumes of antigen and antibody. The tubes were incubated for 15 minutes at 37°C., placed in the refrigerator overnight, and read after centrifugation at high speed. 0 = no precipitate; 1 = small granules; 2 = small floccules; 3 = large floccules; 4 = very heavy precipitate.

Hemolytic Activity of the Stable Hemolysin

Hemolytic Test System.—Preliminary tests for the activity of the stable hemolysin on erythrocytes of various species indicated that the rate of hemolysis (using dilutions of hemolysin from 1:2000 to

1:20,000) was in the following order: rabbit (shortest time for complete hemolysis), horse and sheep. For this reason rabbit erythrocytes were used in the following experiments. The blood was freshly drawn (never more than 3 days old) from normal rabbits. The cells were washed three times with physiological saline solution before use.

Unless otherwise indicated the test system consisted of 0.5 ml. of 5 per cent rabbit erythrocytes plus 0.5 ml. of the hemolysin dilution to be tested. Rapid mixture was carried out in a horizontal tube (Fig. 1), in which holes along one side allowed for introduction of each substance separately. A hollow in the wall of the tube opposite each hole held the fluid until mixing was desired. In order to mix the constituents, the tube was quickly raised to the vertical position, and was shaken continuously with a gentle motion. The amount of fluid left in the cups or adhering to the walls of the vessel above the surface of the mixture was negligible. The end of the tube was constricted to a bore of 0.7 cm. for greater ease in

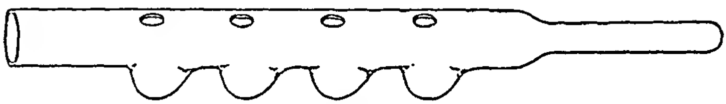

FIG. 1

reading. Hemolysis was read at room temperature by transmitted daylight. Time for complete hemolysis was taken as the end point, although this was not entirely satisfactory, especially in the higher dilutions of hemolysin. However, the reproducibility of results, with the use of this apparatus, seemed to justify the method.

Activity of Highly Purified Stable Hemolysin.—It can be seen from Fig. 2 that complete hemolysis was brought about in a dilution of 1:128,000. (A separate pipette was used for each dilution.) Although partial hemolysis occurred at the next dilution (1:256,000), it was never complete. The stable hemolysin kept in a sterile condition for a period of a year retained its activity. There was no diminution in its activity after 30 minutes in a boiling water bath. After exposure to acid or alkali within what might be called a physiological range (with subsequent neutralization), the activity was not impaired. A comparison of the activity of S. H. which had not been treated, and S. H. which had been exposed to heat and to changes in pH is shown in Fig. 2. A time-dilution curve may be drawn through the points representing the same preparation of S. H. treated in various ways; all of the points fall within the limit of error of the method used.

The crystalline derivative retains hemolytic activity. This activity parallels the activity of S. H., as shown in Table II. We feel that the indicated difference in activity of S. H. and C. S. H. may be due to difficulties in weighing the solid material in order to make solutions of

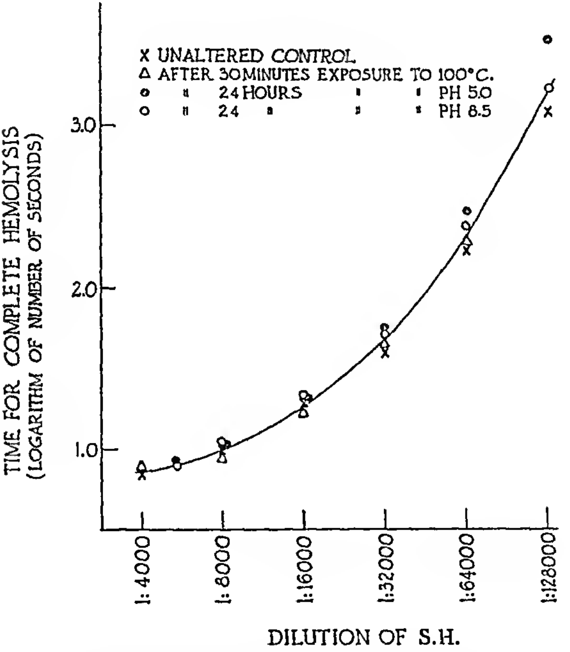

FIG. 2. Stability of the stable hemolysin from β hemolytic streptococci.

known concentrations rather than to an actual difference in activity. The parallel activity of the C. S. H. and the S. H., together with the fact that only the S. H. is serologically active, seems to indicate that those groups in the molecule responsible for serological activity are not those concerned in hemolysis.

In order to determine the nature of the hemolytic reaction, a number of observations were made with the use of the Evelyn colorimeter (11). The progress of the reaction was followed by a decrease in transmissibility of light through the mixture. The transmitted light falls on a photoelectric cell; readings were made on a millivoltmeter. The time-millivolt curve (indicative of time-hemolysis relationship) was S-shaped. That is, after the initial delay, hemolysis proceeded rapidly. The rate then decreased again near the end point of the reaction. For example, when a dilution of 1:150,000 of S. H. acted on 15 ml. of 1 per cent rabbit erythrocytes, there was a lag phase of 2 minutes. Hemolysis then proceeded rapidly, reaching almost complete hemolysis in 10 minutes. The rate then decreased, coming to a constant (complete hemolysis) in 40 minutes. With higher concentrations of stable hemolysin the lag was shorter, as was the time for complete hemolysis. With higher dilutions, the lag was longer, the maximum rate of reaction was less and complete hemolysis was never reached.

These observations show that the stable hemolysin can be classed with the simple hemolysins for which the S-shaped reaction curve is characteristic. The kinetics of simple hemolysins which give such curves have been discussed by Ponder (12).

The Lack of Specific Neutralization of Stable Hemolysin by Immune Sera.—When S. H. is allowed to remain in contact for 30 minutes at 20°C. with immune serum *versus* the homologous or heterologous type of β hemolytic streptococcus, with concentrated antistreptolysin,[1] or with normal rabbit serum, some diminution in the titer of the hemolysin is observed. However, this inhibition of hemolysis was no greater with the immune sera than with the normal serum. After this length of contact no precipitation by the immune serum is visible, and it might well be questioned whether the hapten-antibody reaction had occurred in this time. Therefore, a hemolysis test was performed after the second stage of the precipitation reaction had set in; that is, the precipitation test was carried out in the usual way: dilutions of S. H. were set up with homologous antiserum (in duplicate), with antiserum to a heterologous type, with normal rabbit serum, and with saline. After 15 minutes in the 37°C. water

[1] Obtained from Dr. Homer F. Swift of The Rockefeller Institute.

bath, the mixtures were allowed to stand overnight in the refrigerator. After centrifugation, a marked precipitate was visible with S. H. plus immune serum (heterologous as well as homologous) and not with normal serum nor with saline. A subsequent hemolysis test showed no more inhibition by the immune sera (in the presence of the precipitate) than by normal serum. In the duplicate precipitin test with the homologous serum, the supernatant in each tube was removed to a corresponding tube in another series, and the remaining precipitate was washed once with saline. In a hemolysis test carried out on these two series, the supernatant showed the same degree of hemolysis as the corresponding precipitate plus supernatant; the precipitate alone showed no hemolysis. In summary, anti-β hemolytic streptococcal serum (whether of the homologous or heterologous type) does not inhibit hemolysis by S.H. to any greater extent than normal serum (whether the hapten-antibody reaction has proceeded to the first or second stage). This is in contrast to the very effective neutralization of the oxy-labile hemolysin by immune sera (3, 13).

Leucocidic Activity of the Stable Hemolysin and Its Crystalline Derivative

As it is known that intact cells of streptococci are often leucocidic' the stable hemolysin and its crystalline derivative were tested for their action on leucocytes. The bioscopic method of Neisser and Wechsberg (7) was used. This method depends on the ability of normal leucocytes to reduce methylene blue.

Methods and Materials.—A leucocyte suspension was obtained according to the method of Mudd, Lucké, McCutcheon and Strumia (14) by introducing 200 ml. of sterile saline into the peritoneal cavity of a normal rabbit. 4 hours later the suspension of leucocytes which had accumulated was drawn out through a large needle. It was found that a suspension of leucocytes of at least 10,000 per c. mm. was necessary to bring about the reduction of methylene blue in an appropriately short time. After longer time intervals, the cells settled so that reduction was not uniform throughout the tubes. The cells were left in the peritoneal fluid to keep them in suspension. When it was necessary to concentrate the suspension it was centrifuged for 30 seconds, and enough of the comparatively clear supernatant fluid was removed to make the concentration desired. A control was included in each series, using clear supernatant fluid (peritoneal fluid after intense centrifugation) to determine the effect of this substance on methylene blue. Other controls

The boiling point elevation of a solution of S. H. (100 gm. per 1000 ml.) was found to be between 0.023° and 0.025°C., indicating a molecular weight of the order of 2000; that of C. S. H., 0.075°C., indicating a molecular weight of 720. The S. H. fraction contains phosphorus in the form of phosphoric acid to the extent of 1.37 per cent (colorimetric), and nitrogen (Kjeldahl) to the extent of 1.87 per cent. Thus the nitrogen-phosphorus atomic ratio is 3:1. The minimum molecular weight based on phosphorus is 2260. As boiling point measurements have shown the molecular weight to be of the order of 2000, the actual

TABLE III

Nitrogen and Phosphorus Content of Stable Hemolysin and Molecular Weights Based on Phosphorus

S. H. derived from Strain No.	Phosphorus	Nitrogen	N/P atomic ratio	Molecular weight
	per cent	*per cent*		
1685 cells	1.34	1.88	3.11	2314
1685 washings	1.40	1.92	3.04	2215
1896 cells	1.35	1.84	3.02	2297
1896 washings	1.40	1.87	2.96	2215
S43G cells	1.32	1.87	3.14	2349
1685G cells	1.36	1.84	3.00	2280
O90R cells	1.40	1.84	2.91	2215
F132 cells	1.41	1.89	2.97	2200
		Averages.....	3.02	2260

Cells = S. H. isolated from whole streptococci.

Washings = S. H. isolated from plate washings of mucoid strains of streptococci, from which the organisms had been removed.

A substance of similar solubility could not be isolated from lyophile-dried broth.

molecular weight must be 2260. As there are 3 atoms of nitrogen to 1 of phosphorus, the actual molecular weight based on nitrogen is again the same within the accuracy of the analyses (minimum molecular weight based on nitrogen = $749 \times 3 = 2247$).

The definite ratio of 3 nitrogen atoms to 1 phosphorus atom is further evidence of the high degree of purity of S. H. The phosphorus and nitrogen content of S. H. from eight different sources, and the molecular weight based on these analyses are given in Table III. The

nitrogen-phosphorus ratio is about 3:1 in each case, and indicates that the eight different preparations of S. H. are probably identical, regardless of the strain from which they were isolated.

The stable hemolysin also contains carbon 39.87 per cent, hydrogen 6.54 per cent and oxygen. Its tentative molecular formula obtained by per cent analysis is $C_{75}H_{143}O_{71}N_3P$.

In the formation of C S. H., which is accomplished by heating with NaOH, the nitrogen and phosphorus are lost, leaving only C, H and O.

FIG. 3. Crystalline stable hemolysin photographed through crossed Nicol prisms. × 150.

The nitrogen is given off in the form of ammonia, and is therefore probably acid amide nitrogen. It is evident that during the loss of the N and P, the S. H. has been split into three smaller molecules which are identical (constant composition of C. S. H. after repeated recrystallizations), as three times the molecular weight of C. S. H. plus three times the atomic weight of nitrogen, plus the atomic weight of phosphorus approximates the molecular weight of S. H. (720 × 3 + 42 + 31 = 2233). This molecular weight was confirmed,[2] using a

[2] Confirmation by Dr. Charles G. Grosscup.

modification of the thermoelectric vapor pressure apparatus as introduced by Hill (15); a molecular weight of 2070 ± 300 was reported.

Further work is in progress on the chemical structure of the two compounds.

C. S. H. is one of the few toxic bacterial substances which have been obtained in a crystalline form (16). A photomicrograph of C. S. H. taken through crossed Nicol prisms is shown in Fig. 3. Characteristic crystals are peg-shaped.

Other Properties of Stable Hemolysin and Crystalline Stable Hemolysin

Saturation of homologous antiserum with S. H. does not remove phagocytosis-promoting nor agglutinating antibodies (10).

C. S. H. has been shown to be extremely toxic for mice and rabbits. Further work on the toxicity of and pathological changes produced by C. S. H. and S. H. in animals is being carried out.

SUMMARY

1. A chemically pure hemolysin-leucocidin has been isolated from β hemolytic streptococci, but not from other species of bacteria studied.

2. It does not give rise to antibodies, but precipitates immune sera against hemolytic streptococci, and is therefore a hapten.

3. A highly purified sample of S. H. up to a dilution of 1:128,000 hemolyzes red blood cells. Its hemolytic activity is not specifically neutralized by antiserum *versus* β hemolytic streptococci. It is leucocidic in that it inhibits the reduction of methylene blue by leucocytes.

4. The hemolysin-leucocidin is stable to oxygen, to heat and to moderate changes in hydrogen ion concentration. Its chemical structure has been determined in part. Its molecular weight is 2260.

5. A crystalline derivative has been isolated as the sodium salt from the hemolysin-leucocidin. As the free acid it has a molecular weight of 720. Its hemolytic and leucocidic activity parallels that of S. H., although it is not serologically active. It possesses a high degree of toxicity for mice and rabbits.

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HYDROGEN ION CONCENTRATION IN EXUDATES OF PNEUMOCOCCUS INFECTION

By WILLIAM H. KELLEY, M.D., E. N. SCADRON, M.D., AND B. M. SHINNERS, M.D.

(From the Department of Medicine of the Duke University School of Medicine, Durham, North Carolina)

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Lord (1, 2) has reported that exudates of pneumococcus infection show hydrogen ion concentrations greater than the blood serum by comparative measurement. With the dialysis method he was usually able to demonstrate acidity of marked degree in pneumonic sputum, empyema pus, and in exudates taken at autopsy from the solidified lungs of fatal cases of lobar pneumonia and from those of dogs with experimental pneumococcus pneumonia. In confirmation of Lord's observations, Takahashi (3) showed with electrometric methods that in pneumococcus empyema, the hydrogen ion concentration of the pus becomes progressively increased from approximately pH 7.00 at the onset, to values as great as pH 5.71 in the later stages. Takahashi found in addition that the empyema pus contained only 0.05 to 0.02 per cent of sugar.

Physicochemical changes in inflammation were first described by Opie (4) who titrated a decrease in alkalinity of exudates from the pleural cavities of dogs previously injected with irritants. Lord (1, 2), Koldajew (5), Gollnow (6), and Menkin (7) have with different methods found acidity in exudates from infections due to a variety of bacteria. By further studies of inflammation, Kempner (8) has shown that in the fluid of cantharides skin blisters, carbonic and lactic acids become increased in concentration with the aggregation of leucocytes, while the base and sugar are proportionately diminished in comparison with the blood. Light from another angle has been thrown upon the origin of these physicochemical changes by Menkin (9) and others. From the injection of dyes, the latter workers have adduced evidence to indicate that in focal inflammation, capillary permeability is increased, whereas diffusion from the lesion into the surrounding tissues and blood stream is reduced by the coagulation of fibrin and plasma in the capillaries and lymphatics. Thus, it appears that localized inflammation generally is characterized by an accumulation of acids with a corresponding depletion of base and

sugar, which proceeds from the metabolic activities of leucocytes, or leucocytes and bacteria, in an enviroment partly isolated from the rest of the body.

This paper is a report of observations on the hydrogen ion and sugar concentrations in exudates of pneumococcus infection. The exudates were obtained from the solidified lungs of dogs with pneumonia and from dermal infection in rabbits produced experimentally with the pneumococcus. Whereas Lord (1, 2) examined exudates secured post mortem, our materials were collected with biopsy methods in an effort to determine more nearly the range of hydrogen ion and sugar concentrations which occur within these lesions *in vivo*. In this way we have sought to compare the magnitude of the physicochemical changes within these primary infections with those described by Takahashi (3) in secondary empyema due to the pneumococcus.

Materials and Methods

Pneumonic Exudate.—Pneumonia was produced in normal dogs with a highly virulent strain of Type I Pneumococcus after the method of Terrell and Robertson (10). The course of the infection was followed with temperature readings, leucocyte counts, blood cultures, and x-ray examinations. Animals given artificial pneumothorax, received injections of 250 to 300 cc. of air over the affected lung at 20 to 24 hours and again at 32 to 36 hours. At stated intervals after inoculation, the animals were given 20.0 to 25.0 mg. per kilo of body weight of sodium amytal intravenously and a biopsy was then done. Through an incision across the costal cartilages, a consolidated lobe was excised and blotted dry of blood escaping from the open vessels. The pneumonic exudate was expressed from openings in the distal pleural surface and collected in cold dry dishes.

Exudate from Dermal Pneumococcus Infection.—Dermal infection was produced in large normal rabbits with highly virulent Type I pneumococci by the technic of Goodner (11). At given intervals during the course of the infection, fluid for examination was aspirated from the lesions with syringe and needle.

pH Determinations.—Specimens of exudate were covered with oil and kept on ice until the pH measurements could be completed. Determinations on the pneumonic exudate[1] from dogs were made electrometrically with a glass electrode known to be accurate to 0.10 of absolute pH and within 0.05 pH between different readings. Determinations on the exudate from dermal infection in rabbits were made with platinum electrodes and quinhydrone with approximately the same accuracy.

Sugar Determination.—On specimens of blood and exudate collected simultane-

[1] Kindness of Dr. T. Coolidge of the Department of Biochemistry.

ously, sugar determinations were done in duplicate by the micro method of Hagedorn-Jansen with cotton filters.

Pneumonic Exudate from Dogs

The course of the pneumococcus pneumonia in typical instances in dogs of these experiments is shown for reference in Chart 1. It can be seen that the infections were severe with bacteremia as a rule.

It is noteworthy that the exudate expressed from the pneumonic

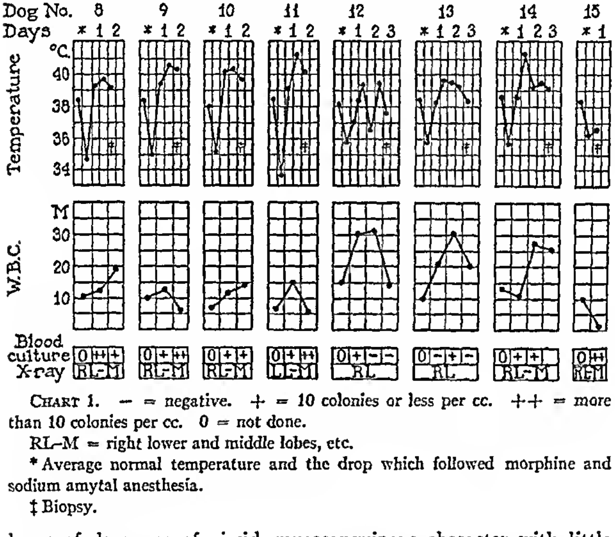

CHART 1. — = negative. + = 10 colonies or less per cc. ++ = more than 10 colonies per cc. 0 = not done.

RL-M = right lower and middle lobes, etc.

* Average normal temperature and the drop which followed morphine and sodium amytal anesthesia.

‡ Biopsy.

lungs of dogs was of viscid, mucosanguinous character with little fluid and a high content of fibrin and cellular materials comprised largely of phagocytes and erythrocytes in various states of preservation. The exudates also contained pneumococci in numbers between 6 hundred thousand and 8 million per cc. as determined from cultures of serial dilutions.

The results of the hydrogen ion and sugar determinations upon pneumonic exudate from fourteen dogs are presented in relation to

the duration of the infection and the general condition of the animals at the time, in Table I. The findings on the exudate from four dogs which received artificial pneumothorax are combined by reason of their similarity with those on the exudate from untreated animals. Included also for comparison are the results of sugar determination on the pneumonic exudate and blood from three animals which had died of the infection.

TABLE I

The Hydrogen Ion and Sugar Concentrations in Exudate from the Consolidated Lungs of Dogs with Experimental Pneumonia

Dog No.	Condition of animal	Duration of infection	pH of exudate	Sugar		Sugar deficit in exudate
				Blood	Exudate	
		hrs.		*mg. per cent*	*mg. per cent*	*per cent*
4	Moderately ill	36	—	178–184	72–80	58.1
6	" "	48	6.80	152–153	81–81	46.5
8	Severely "	42	7.15	124–130	56–60	54.3
11	" "	48	6.75	86–93	43–54	44.8
12	Recovering	64	7.16	127–134	110–116	13.4
14	Extremely ill	66	6.73	66–74	47–53	28.5
15	" "	16	7.18	54–60	17–24	64.0
5*	Severely "	48	—	63–72	74–74	+9.6
7*	" "	36	7.07	102–106	105–110	+3.3
9*	" "	44	6.75	101–103	91–95	8.8
10*	" "	40	6.75	129–133	86–93	31.6
1	Dead, 15 min.	44	—	54–58	36–41	31.2
2	" 30 "	30	—	50–57	26–32	56.5
3	" 4–6 hrs.	24	—	38–43	15–20	32.0

— = not done.

* Artificial pneumothorax therapy.

The hydrogen ion concentration of the exudate from three of six untreated animals and from three others which received artificial pneumothorax was definitely greater than is found in the blood of living dogs. The pH of the exudates from three untreated animals approached the lower limits of normal for the blood. The pH of the exudates varied between 7.18 and 6.73 and bore no apparent relationship to the duration of the infection, or to the general condition of the animals employed in the experiments.

The sugar content of the pneumonic exudate was significantly lower than that of the blood in all dogs except three of the four dogs treated with artificial pneumothorax, in which the values for exudates and blood were practically equal. Parallel to the observations of Lord (1, 2) upon the increase of acidity in pneumonic exudates taken at autopsy, the sugar concentrations were below normal in the blood and still further reduced in the exudates from animals which had died of the infection. Difference in sugar content between the exudate and blood could not be correlated with the pH of the exudate, the duration of the infection, or the condition of the animal in the experiments. Blood sugar levels in living animals were high or low depending upon

TABLE II

The Hydrogen Ion and Sugar Concentrations in Exudate from Dermal Pneumococcus Infection in Rabbits

Rabbit No.	Condition of animal	Duration of infection	pH of exudate	Sugar		Sugar deficit in exudate
				Blood	Exudate	
		hrs.		*mg. per cent*	*mg. per cent*	*per cent*
4	Moderately ill	18	6.80	131–140	67–72	47.8
5	" "	18	6.76	105–112	33–42	65.4
	Extremely "	30	6.66	83–88	51–60	35.1
6	Moribund	30	6.78	39–48	9–15	72.4
7	Moderately ill	36	6.87	120–129	80–85	33.4
	" "	60	6.81	112–112	40–45	62.0

whether or not they had been too ill to eat the food which was freely provided for all.

Exudates from Dermal Infection in Rabbits

As an example of practically non-sanguinous exudate from a primary pneumococcus infection, the serous fluid aspirated from typical lesions of dermal infection in rabbits was examined with the results given in Table II.

It can be seen that the hydrogen ion concentration of the exudate from the dermal infection was regularly increased beyond values found in the blood of living rabbits. Likewise, the sugar content of

the exudate was in each instance considerably lower than that of the blood. However, the hydrogen ion concentration of the exudate which varies between pH 6.87 and 6.66, was not always proportional to the difference in sugar content between the exudate and blood under the condition of our experiments.

SUMMARY

The hydrogen ion concentration in the lesions of experimental pneumococcus infection has been estimated directly by pH determinations on exudates from living animals. For indirect evidence of an increase in hydrogen ion concentration within the lesions, the difference in sugar content between exudate and blood from animals with pneumococcus infection has been measured.

With sanguinous exudate from the consolidated lungs of dogs with experimental pneumococcus pneumonia, the findings were not always consistent, but usually there was either direct or indirect evidence of increased hydrogen ion concentration. The physicochemical changes in exudate from animals treated with artificial pneumothorax showed no important differences from those in other specimens. In concurrence with Lord's (1, 2) observation of increased acidity in pneumonia exudate obtained at autopsy, sugar concentrations, which are low in the blood, were markedly reduced in exudates from animals which had died of the infection.

Serous exudates from dermal pneumococcus infection in rabbits uniformly showed definite acidity by both direct and indirect methods of estimation. The hydrogen ion concentrations in exudate from dermal pneumococcus infection in rabbits varied between pH 6.87 and 6.66 but were not always proportional to the difference in sugar concentrations between the exudate and blood. While these hydrogen ion concentrations are similar to those attained in the pneumonic exudate from dogs, they are of lesser magnitude than those which Takahashi (3) has described in the pus of secondary empyema due to the pneumococcus.

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THE EFFECTS OF ACIDITY UPON THE GROWTH OF PNEUMOCOCCUS IN CULTURE MEDIA CONTAINING PROTEINS

By WILLIAM H. KELLEY, M.D.

(From the Department of Medicine of the Duke University School of Medicine, Durham, North Carolina)

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In a preceding communication (1) we have reviewed the accumulated evidence and presented additional data to indicate that a definite degree of acidity occurs in the lesions of pneumococcus infection. The rôle of the acidity in the lesions of pneumococcus infection is not as yet clearly understood. That acidity *in vitro* exerts a bacteriostatic effect upon the pneumococcus was proven by the studies of Dernby and Avery (2), and Lord and Nye (3, 4), which showed that in broth, hydrogen ion concentrations greater than pH 6.80 not only inhibit growth but within a short time produce death of this bacterium. However, Morgan and Avery (5) have shown that the range of hydrogen ion concentrations at which pneumococcus growth can be initiated may be extended to a degree of acidity as great as pH 6.2 in broth by the addition of fresh unheated plant tissues. Possible evidence that acidity may be less active in inhibiting the growth of pneumococcus in animal tissue fluids is afforded by the observations of Felton and Dougherty (6) who were able to maintain growth of this microorganism in milk at pH 5.00, when adjusted with hydrochloric acid.

The present paper is a report of studies on the effects of acidity upon the growth of the pneumococcus in culture media containing tissue fluids or animal proteins.

Materials and Methods

Cultures.—The findings reported were obtained with a standard strain of Type I Pneumococcus which was maintained at maximal virulence by mouse passage during the course of the experiments.

Culture Media.—Beef infusion broth was prepared with neopeptone (Difco),

10.0 gm.; sodium chloride, 5.0 gm.; and the infusion from 1.0 pound of lean beef in a volume of a liter, in the usual manner.

The animal sera used were secured fresh from their sources. Other proteins were obtained commercially. Materials containing proteins were sterilized by Berkefeld filtration.

Hydrogen Ion Concentration.—The hydrogen ion concentration of the culture media was adjusted electrometrically with platinum electrodes and quinhydrone. Final hydrogen ion concentrations of cultures were measured in the same way. The readings were accurate within 0.10 of absolute pH and within 0.05 pH of each other.

The Initiation of Pneumococcus Growth in Serum Broth Adjusted to Acid Hydrogen Ion Concentration with Hydrochloric and Acetic Acids

As a method of ascertaining the effect of acidity upon the growth of pneumococcus in the presence of animal fluids, we have employed the technic described by Dubos (7) to determine the number of these bacteria required to initiate growth in broth to which fresh serum was added, and for comparison, in plain broth prepared with peptone freed of growth inhibitory substances after the method of Dubos (7). Inasmuch as the acids occurring in cultures and in the inflammatory lesions of the pneumococcus are well known to be of the organic variety, acetic as well as hydrochloric acid has been used to adjust the culture media to acid hydrogen ion concentrations. The amount of inoculum which was required to initiate growth of Type I Pneumococcus in plain broth and in broth containing 50 per cent of human serum, when adjusted to varying hydrogen ion concentrations with acetic or hydrochloric acid, is shown in Table I.

The bacteria from 8 hour plain broth cultures of virulent Type I Pneumococcus were removed by centrifugation and resuspended in an equal volume of the media to be tested for growth inhibition. From the new suspension, 0.5 cc. was used to inoculate eight tubes containing 4.5 cc. each of the same media after the usual method of serial dilution. The approximate number of pneumococci were determined by culturing 0.5 cc. of each of the two highest dilutions in blood agar pour plates. After 24 hours at 37.5°C., the cultures were examined for density of growth which was proven to be pneumococcus by subculture on blood agar.

It can be seen in Table I that in plain broth at pH 7.8 a minimal number of cells were sufficient to initiate the growth of pneumococcus. In plain broth adjusted to pH 7.0 with either acetic or hydrochloric

The Amount of Inoculum Required to Initiate Growth of Type I Pneumococcus in Plain Broth and in Broth Containing [illegible] Cent of Fresh Human Serum, When Adjusted to Varying Hydrogen Ion Concentrations with Acetic or Hydrochloric Acid

	pH	Density of growth after 24 hrs. at 37.5°C.							
Dilution of inoculum		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Plain broth	7.8	++++	++++	++++	++++	++++	++++	++++	+++
Plain broth adjusted with HCl acid	7.0	++++	++++	++++	++++	++	–	–	–
	6.5	–	–	–	–	–	–	–	–
	6.0	–	–	–	–	–	–	–	–
Plain broth adjusted with acetic acid	7.0	++++	++++	++++	++++	+	–	–	–
	6.5	–	–	–	–	–	–	–	–
	6.0	–	–	–	–	–	–	–	–
50 per cent serum broth adjusted with HCl acid*	7.0	++++	++++	++++	++++	++++	++++	++++	++++
	6.5	++++	++++	++++	++++	++++	++++	++++	++++
	6.0	++++	++++	++++	++++	++++	++++	++++	++++
	5.5	++++	++++	++++	++++	++++	++++	++++	++
	5.0	++++	++	–	–	–	–	–	–
50 per cent serum broth adjusted with acetic acid*	7.0	++++	++++	++++	++++	++++	++++	++++	++++
	6.5	++++	++++	++++	++++	++++	++++	++++	++
	6.0	++++	++++	++++	++	–	–	–	–
	5.5	++	–	–	–	–	–	–	–
	5.0	–	–	–	–	–	–	–	–

++++ = turbid growth.
+++ = cloudy "
++ = many colonies on blood agar, slightly cloudy.
+ = few " " " "
– = no " " " "

* Serum broth at pH 5.0 showed a small amount of precipitate.

substances were not examined for their ability to protect the pneumococcus against the effects of acidity. However, preliminary observations indicated that serum albumin or globulin of the horse, casein of milk, and vegetable mucoid when added to broth in appropriate concentration, support pneumococcus growth in the presence of acidity.

The Growth Rate of Type I Pneumococcus in Serum Broth at Acid Hydrogen Ion Concentrations

With the above results in mind, it seemed of interest to compare the rate of growth of pneumococcus at favorable and unfavorable

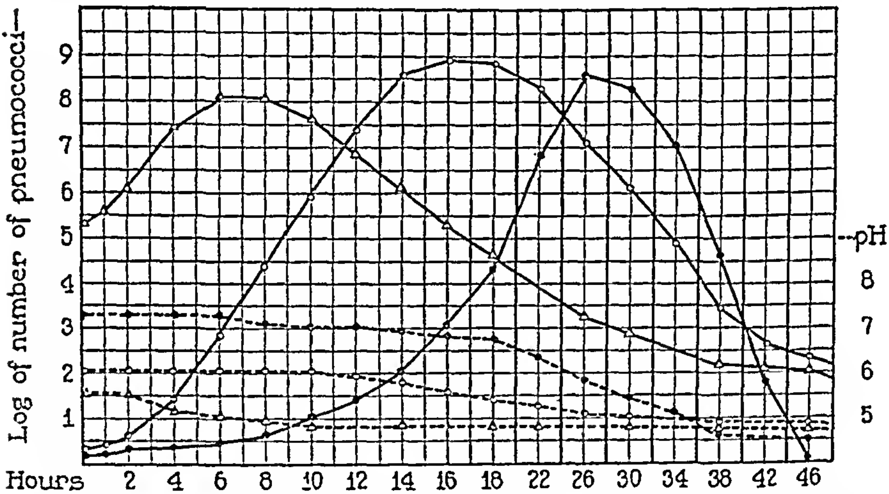

CHART 1. ● = 0.5 per cent dextrose broth. ○, △ = 0.5 per cent dextrose broth plus 50 per cent swine serum.

degrees of acidity in serum broth with that which occurs at the optimal hydrogen ion concentration in broth alone. Accordingly the growth rate of Type I Pneumococcus in 50 per cent swine serum, 0.5 per cent dextrose broth at pH 6.5 and at pH 6.0, and in 0.5 per cent dextrose broth alone at pH 7.8, has been determined with the results given in Chart 1.

After the method developed by Chesney (8), 50.0 cc. amounts of the media employed were adjusted with acetic acid to the stated hydrogen ion concentrations and brought to 37.5°C. These portions of media were inoculated with measured

quantities of a growing 8 hour broth culture of Type I Pneumococcus and kept at 37.5°C. At stated intervals samples were removed for pH determinations and for enumeration of the bacterial cells by pour plate cultures of serial dilutions. To insure completion of the growth cycle of the cultures, 0.5 per cent dextrose was added to all media.

From Chart 1 it can hc seen that the growth rate of pneumococcus in the serum dextrose hroth at pH 6.5 exceeded that in the dextrose broth alone at pH 7.8. Howevcr, in serum dextrose broth at pH 6.0 a larger amount of inoculum was required for initiating pneumococcus growth and the greatest number of cells attained at the height of the cycle was somewhat less than that in either serum dextrose broth at pH 6.5 or dextrose broth alone at pH 7.8. In serum dextrose broth at either pH 6.5 or 6.0, the phases of maximal growth and decline in the growth curves were prolonged in comparison with those in dextrose broth alone. Lord and Nye (9) found that fresh serum inhibits autolysis of pneumococcus and it is of interest that these bacteria were proven by subculture to remain viable in the serum dextrose broth cultures for as long as 60 to 90 hours, while in the dextrose broth alone no living cells could be demonstrated after approximately 42 hours at 37.5°C. The final hydrogen ion concentrations attained in serum dextrose broth cultures varied between pH 5.4 and 5.2, and those in dextrose broth alone lay between pH 5.1 and 5.0. Variations in the decline period of growth and tbe final hydrogen ion concentration of pneumococcus cultures in the serum dextrose broth were apparently influenced by factors beyond the scope of this report, which will be dealt with in a separate communication.

SUMMARY

In the presence of animal fluids or their protein constituents, Type I Pneumococcus survived and multiplied at acid hydrogen ion concentrations which in the plain broth were bactericidal for these organisms. Minimal numbers of these cells readily produced pneumococcus growth in serum broth when adjusted at hydrogen ion concentration as great as pH 5.5 with hydrochloric, or to pH 6.5 with acetic acid. Growth of the pneumococci could he demonstrated in serum broth adjusted to pH 5.0 with hydrochloric, or to pH 5.5 with acetic acid although at these hydrogen ion concentrations, large amounts of

inoculum were necessary. Similar results were obtained with broth to which certain animal proteins were added and in serum broth which was previously heated by autoclave at 20 pounds pressure for 20 minutes.

Pneumococcus growth proceeded at a more rapid rate in serum dextrose broth at pH 6.5 than in dextrose broth alone at the optimal hydrogen ion concentration of pH 7.8. At pH 6.0 large numbers of pneumococci failed to produce the same amount of growth in serum dextrose broth as at pH 6.5 or in dextrose broth alone at pH 7.8. It is of interest that in serum dextrose broth cultures, the stationary and decline phases of pneumococcus growth were prolonged and cell death delayed in comparison with cultures in dextrose broth alone.

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BLOOD PLASMA PROTEIN REGENERATION AS INFLUENCED BY FASTING, INFECTION, AND DIET FACTORS

Variable Reserve Stores of Plasma Protein Building Material in the Dog

By S. C. MADDEN, M.D., W. E. GEORGE, G. S. WARAICH, M.D., and G. H. WHIPPLE, M.D.*

(From the Department of Pathology, The University of Rochester School of Medicine and Dentistry, Rochester, New York)

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This paper continues the study of blood plasma protein production in dogs as influenced by various factors. Plasma protein production is measured by determination of the protein removed in daily plasmapheresis (removal of whole blood and return of washed red cells suspended in a saline solution). The factors known to influence plasma protein production are controlled while this measurement is being made day after day, week after week. Some of these factors are the dietary intake of protein, the reserve store of plasma protein building material, the concentration of the circulating plasma protein, and the clinical state of health of the animal (freedom from infection, etc.). Data bearing on all these factors have been published in previous reports (3, 10, 7, 5) and further experiments particularly concerning the reserve store are presented in this communication. The details of the procedure used are reviewed below (Methods).

The presence of a *reserve store* of plasma protein building material in the normal dog is well established. Its amount varies in different dogs. It may be as low as 10 gm. or as high as 60 gm.—compared with about 30 gm. plasma protein, the mass circulating in this type of normal dog (10 to 13 kg.). To a great extent these wide variations are dependent upon the preceding dietary intake. After weeks on a diet low in protein the reserve store will be low, and following periods

* J. V. Sullivan, M.D., assisted in some of the preliminary experiments.

of liberal protein feeding a large reserve store will be accumulated. This reserve store may be even larger if a period of plasma depletion has preceded the period favorable to storage (Table 1, below).

The *time required for the depletion of this reserve store* varies directly with the amount of the store and is usually from 2 to 6 weeks (see Table 1). In the depletion of this reserve store the mere reduction of the circulating plasma protein concentration from a normal level to a concentration slightly above the edema level is no criterion of exhaustion of the store. Reference to Tables 2 and 4 below will show that such a reduction may occur from 1 to 4 weeks prior to complete removal of the reserve store. Therefore, it is essential that the hypoproteinemia be maintained a sufficient length of time (2 to 6 weeks) to demonstrate a *constant* minimal (basal) plasma protein production. To do this a diet must be chosen which will maintain the dog in an apparently normal state for an indefinitely long time. If the basal diet is inadequate no certain baseline can be established. This opinion relative to reserve stores differs from that held by others (8).

This *reserve store* of plasma protein building material we believe plays a rôle in protein metabolism which is only beginning to be understood. Under certain circumstances it can contribute to the building of other body protein, for example hemoglobin. It may participate in and modify the response to certain injuries involving body protein. For example, when a standard sterile abscess is produced in a dog which has been depleted of this reserve protein store the urinary nitrogen does not increase in the striking fashion noted in control normal dogs (Table 3-*a*) (1, 2). Evidently this reserve protein store contributes directly or indirectly to the surplus of eliminated urinary nitrogen observed with abscesses in normal dogs.

Methods

The *reserve store* in these experiments is and has always been (3, 10, 7, 5) determined as follows. After a week's fast the dog is fed a standard basal low protein diet daily as given in the clinical history of each animal. Daily plasmaphereses are done and the total protein removed each week recorded in the tables. An attempt is made to reduce the plasma protein concentration from the normal level of about 6.0 per cent to the depletion level of 4.0 per cent and to hold this depletion level at a very constant figure. At about 3.5 per cent plasma protein concentration edema and clinical disturbances may appear and

at 4.6 per cent some storage of protein in body tissues will occur. The bleedings are carefully estimated to keep the plasma protein concentration close to this optimum figure of 4.0 per cent and it is safe to assume that this level supplies a constant stimulus to the body to produce as much plasma protein as possible on the given diet intake. The red cell hematocrit is maintained at about 50 per cent by the return of suitable amounts of washed red cells. This plasmapheresis is continued steadily until the weekly plasma protein output is a constant or approximately so, and this usually requires 4 to 6 weeks of plasmapheresis (dogs 33-11 and 36-95). An actual example of the method used in calculation of the reserve store may now be given. In dog 36-95, Table 4, if the plasma protein weekly production attributable to the basal diet is assumed to be 20 gm. on the basis of its constancy in periods 4 and 5, the total basal output for the first 5 weeks (including the week of fasting) is 80 gm., but the *total plasma protein actually removed* is 105 gm. The difference equals a reserve store of 25 gm. To allow for that portion contributed by the mass of circulating protein during the reduction of its concentration from 5.34 to 4.04 gm. per cent we may deduct 7 gm. (assuming a constant plasma volume of 550 cc.) and obtain a *net reserve store* of 18 gm.

Adult dogs immunized (Laidlaw-Dunkin) against distemper were used. Their care and feeding have been previously described (5). Certain changes in procedure are here enumerated.

1. Fecal nitrogen determinations were done. As collected the feces were suspended in water and charred by the addition of an equal volume of concentrated sulfuric acid. The quantities thus treated for a complete week were then diluted to volume and nitrogen determined on a pipetted aliquot.

2. Boric acid, 4 per cent aqueous solution, was used to hold the distilled ammonia in solution in performing the Kjeldahl nitrogen determination (11).

3. No glucose was added to the modified Locke's solution used in suspending the washed red cells for injection into the dogs. Previously in this work glucose to a 5 per cent concentration has been used, but such hypertonicity induces crenation of the cells. The donor cells are always freshly prepared and injected within less than 3 hours after their removal from the donor dogs.

4. Large negative nitrogen balances have been recorded for the dogs subjected to plasmapheresis (3, 10, 7, 5). Part of this deficit has been calculated on the assumption in some instances of a fecal nitrogen output of 1 gm. per day. A larger part of the deficit has arisen from failure to account for the nitrogen in *excess* red blood cells injected for the purpose of maintaining a constantly normal hematocrit. All the work reported in this paper was done prior to attempts at exact measurement of red cells injected, and the *recalculation* that has been done probably includes considerable error.

As in previous work we have intended to feed the dogs basal diets containing the minimal protein consistent with a normal existence during prolonged plasmapheresis. The exact diets used are given in the clinical histories. The percentage protein of the various diets is calculated from the following figures: pork liver, 20 per cent, pork kidney, 16.2 per cent; canned tomatoes, 1.2 per

cent; gelatin, granular (Will Corporation), 83.8 per cent (15.1 per cent nitrogen); "protein-free" diet of Cowgill, 0.05 gm. protein (but 0.019 gm. nitrogen) per kilo dog. About 95 per cent of the nitrogen in this latter diet is present in the liver extract vitamin supplement. The protein-free diet was given exactly as described by its originators (8). Except in this latter, the salt mixture used in all diets was that of McCollum and Simmonds (6) without the iron. Plasma volume determinations were done at the end of each week as tabulated.

EXPERIMENTAL OBSERVATIONS

Plasma protein building stores may vary in the same dog under different conditions. It is known that long periods on a low protein diet or short periods of fasting will deplete this reserve. As illustrated

TABLE 1

Reserve Store of Plasma Protein Building Material Determined in 3 Successive Years in Dog 33-11

Period	Diet before depletion	Weight		Plasma protein concentration		Time of deple-tion*	Total reserve protein
		Initial	Final	Initial	Final		
		kg.	*kg.*	*gm. per cent*	*gm. per cent*	*wks.*	*gm.*
1935 (7)	Low protein 9 wks.	11.6	11.2	5.04	4.10	2	11.1
1936 (5)	Kennel 16 wks.	12.5	11.5	6.09	4.13	3	34.1
1937 (Table 2)	Kennel 20 wks.	13.1	11.4	6.50	4.05	6	67.0

* One week of fasting precedes each depletion period—see Table 2.

in Table 1, the first period shows a very low protein store, due in part to the preceding 9 weeks period of potato-bran basal ration (7)—a low protein diet containing only vegetable and grain proteins.

Table 1—the second and third periods are comparable as the dog in 2 successive years had been on the kennel diet (hospital table scraps) for 16 and 20 weeks, had been fasted for 1 week, and then put on the same kidney basal diet for plasma depletion. The protein reserve store in the second period was 34 gm. and one year later 67 gm. This observation suggests that a dog subjected to plasma depletion may subsequently pile up a larger protein reserve store when a favorable diet offers the opportunity for storage.

Tables 2 and 2-*a* show 18 consecutive weeks of plasma depletion in an active dog, clinically normal. The following 12 weeks are recorded

in Tables 3 and 3-*a*. Details relating to diet intake are given under clinical histories below. The first seven periods show a large *reserve store* of plasma protein building material—about 67 gm. plus the unknown amount used up during the first period of protein fasting. From other experiments in preceding years we estimate the basal output as 12 gm. plasma protein each week but even if we place the basal output at 13 to 14 gm. plasma protein per week, the surplus protein store is 50 to 60 gm. and requires 7 weeks for removal. If experimental observations with gelatin or other materials had been begun at the end of 2 weeks initial depletion, the errors due to undepleted reserve would have been large. It is noted that the albumin-globulin ratio falls from 1.1 at the start of the rapid plasma depletion to 0.7 at the end of the 3rd week but rises again as the plasma depletion is less vigorous at the end of the 6th period. This reaction to plasma depletion has been noted before.

Liver basal ration was then used and the liver contains practically all the ingested protein except for small amounts present in the canned tomatoes. Dogs tolerate the liver basal well and remain clinically normal even when plasma depletion is continued for 30 weeks.

Gelatin added to the basal ration during the 11th period causes very little increase in production of plasma protein, a total of 7.7 gm. as the result of feeding 105 gm. gelatin. It is noted (Table 2-*a*) that the *intake* in food nitrogen is increased about 16 gm. and the *output* of urinary nitrogen likewise is increased about 16 gm. giving a positive nitrogen balance of only 1.7 gm.

Gelatin plus tryptophane added to the basal ration during the 16th period gives a very different picture and an increase of plasma protein production of more than 28 gm. or about 4 times the figure for gelatin alone. Evidently gelatin is supplemented by tryptophane and the basal diet so that it is readily built into plasma protein after digestion. In fact the *potency ratio* for this gelatin plus tryptophane is 3.0 as compared with a ratio of about 12 for gelatin alone. This is compared with the potency ratio of the liver in the basal diet of 4.3—that is 4.3 gm. of liver protein as fed will produce 1 gm. of plasma protein. The positive nitrogen balance during period 16 amounts to 8.2 gm.

Tryptophane alone added to the basal diet in the 23rd period (Table 3) shows no response.

The protein-free diet during period 14 gives a plasma protein output of 8.8 gm.—refer to Table 4, periods 6 and 7, for more complete observations.

TABLE 2

Blood Plasma Protein Production as Influenced by Gelatin and Tryptophane

Dog 33-11.

Period 7 days	Diet	Protein intake Total for 7 days	Plasma protein removed Total for 7 days	Protein removed above basal*	Blood plasma Average concentration: Total protein	Blood plasma Average concentration: A/G ratio	R.B.C. hematocrit, average	Plasma volume
		gm.	gm.	gm.	per cent		per cent	cc.
	Kennel				6.50	1.2	47.2	485
1	Dextrose, 350 gm.	0	3.2		5.72	1.1	45.0	468
2	Kidney basal	64	30.0		4.92	1.0	46.5	—
3	Kidney basal	64	27.9		4.63	0.7	48.8	468
4	Kidney basal	55	24.1		4.04	0.8	52.2	—
5	Kidney basal	64	20.5		3.93	0.8	54.0	398
6	Kidney basal	64	13.8		4.06	0.9	52.6	390
7	Kidney basal	64	17.8	67.0	4.05	0.9	51.7	459
8	Liver basal	108	21.2		4.18	0.9	49.8	411
9	Liver basal	109	24.2		4.14	0.9	48.4	450
10	Liver basal	109	23.6		4.27	1.0	50.0	—
11†	Liver basal + gelatin, 105 gm.	197	26.8	7.7	4.26	1.1	52.2	441
12‡	Liver basal	109	26.0		4.19	1.0	52.8	504
13	Liver basal	109	26.9		4.05	1.0	52.0	438
14	Protein-free	4	8.8		3.97	0.9	50.4	461
15	Liver basal	109	22.4		4.08	0.9	50.2	484
16	Liver basal + gelatin, 105 gm. + tryptophane, 2.7 gm.	197	28.3	28.7+	4.35	1.0	52.5	459
17	Liver basal	109	33.2		4.30	1.0	53.6	462
18	Liver basal	109	39.2		4.35	0.9	53.4	—

* Estimated basal output on kidney basal diet = 12 gm. plasma protein per week and on liver basal = 24 gm. per week.

† 8 day period recalculated on basis of 7 days.

‡ 6 day period recalculated on basis of 7 days.

Tables 3 and 3-*a* continue the experimental life history of dog 33-11. Period 19 presents some interesting departures from normal and we cannot give a satisfactory explanation as yet. Possibly experiments in progress may make clear some of these obscure points. On the 2nd day of the 19th period, the donor cells were probably damaged

by overheating. When introduced into dog 33-11 there was *prompt hemolysis*, the dog was clinically abnormal but not seriously shocked and there was hemoglobinuria for about 24 hours. 3 days later the

TABLE 2-*a*

Weight and Nitrogen Balance

Dog 33-11.

Period 7 days	Diet	Weight	Nitrogen balance					
			Intake		Output			Intake minus output
			in diet	in excess R.B.C. injected	in plasma	in urine	in feces	
		kg.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
	Kennel	13.1						
1	Dextrose, 350 gm.	11.7	0.0	−2.5	0.5	17.7	0.5	−21.2
2	Kidney basal	11.7	10.2	8.1	4.9	13.4	2.5	−2.5
3	Kidney basal	11.7	10.2	2.1	4.6	12.9	2.2	−7.4
4	Kidney basal	11.5	8.8	5.3	4.0	12.5	2.1	−4.5
5	Kidney basal	11.4	10.2	2.9	3.4	11.5	2.0	−3.8
6	Kidney basal	11.4	10.2	3.1	2.3	10.4	1.8	−1.2
7	Kidney basal	11.4	10.2	−1.6	2.9	10.7	1.8	−6.8
8	Liver basal	11.3	17.3	3.1	3.5	9.6	3.1	4.2
9	Liver basal	11.4	17.5	2.1	4.0	9.7	2.5	3.4
10	Liver basal	11.5	17.5	3.1	3.9	10.9	2.4	3.4
11*	Liver basal + gelatin, 105 gm.	11.6	33.4	2.3	4.4	26.6	3.2	1.5
12†	Liver basal	11.6	17.5	0.9	4.5	11.2	2.7	0.0
13	Liver basal	11.7	17.5	0.8	4.4	10.5	3.5	−0.1
14	Protein-free	11.5	1.4	−0.7	1.4	7.2	1.1	−9.0
15	Liver basal	11.5	17.5	6.1	3.7	10.3	2.2	7.4
16	Liver basal + gelatin, 105 gm. + tryptophane, 2.7 gm.	11.6	33.7	5.2	4.6	24.4	1.7	8.2
17	Liver basal	11.7	17.5	2.0	5.4	11.8	4.3	−2.0
18	Liver basal	11.8	17.5	4.0	6.5	13.0	2.4	−0.4
Totals			268.1	46.3	68.9	234.3	42.0	−30.8

* 8 day period recalculated on basis of 7 days.

† 6 day period recalculated on basis of 7 days.

dog was back to normal, although the plasma was still icteric. Food consumption was normal and there was no significant fall in red cell hematocrit as extra red cells were given by vein. Excess urinary nitrogen amounted to 5 or 6 gm.

This whole reaction is of interest because there is a large excess of

plasma protein produced during periods 19 to 21—an excess of 52 gm. plasma protein over and above the basal output of 24 gm. per week. This *excess* of plasma protein may be related to one or more of several factors—the large surplus of globin released from hemoglobin in the circulation; the clinical shock and related tissue or cell injury; a "carry over" from the period of very abundant plasma protein production

TABLE 3

Blood Plasma Protein Production as Influenced by Hemolysis and by Abscesses Dog 33-11.

Period 7 days	Diet	Protein intake Total for 7 days	Plasma protein removed* Total for 7 days	Blood plasma Average concentration		R.B.C. hematocrit, average	Plasma volume
				Total protein	A/G ratio		
		gm.	gm.	per cent		per cent	cc.
19	Liver basal (hemolysis)	109	51.1	4.29	0.8	51.0	484
20	Liver basal	109	43.1	4.22	0.8	50.5	488
21	Liver basal	104	30.5	3.98	0.8	49.5	457
22†	Liver basal	109	26.3	3.92	1.0	49.5	511
23	Liver basal + tryptophane, 4.2 gm.	109	21.0	4.38	0.8	50.9	482
24	Liver basal	109	17.6	4.22	0.9	50.2	486
25	Liver basal	109	19.2	4.27	0.9	51.3	505
26	Fasting + abscesses	0	15.5	4.76	0.6	51.1	—
27	Liver basal	108	17.1	4.95	0.6	49.5	452
28	Liver basal	108	23.5	4.52	0.8	54.3	428
29	Liver basal	109	19.7	4.24	0.9	52.7	497
30	Liver basal	107	15.8	4.21	0.8	50.4	458

* Estimated basal output on liver basal diet = 24 gm. plasma protein per week.
† 8 day period recalculated on basis of 7 days.

due to gelatin plus tryptophane 3 weeks previously. More experiments are required to give a complete answer.

Tables 3 and 3-*a* (period 26) show an unexpected reaction with plasma protein production much above the 4 to 8 gm. usually obtained during fasting (10, 7). We believed from previous experiments (5) that *abscesses* lessened the output of plasma protein during periods of liver feeding and we suspected that the fasting output with abscesses might be zero or even show a rapid fall in plasma protein con-

centration. Therefore the plasma protein concentration was not held at the usual level of 4.0 per cent but permitted to rise as high as 4.76 per cent. This rise confused the picture and we cannot argue about the surplus plasma protein (15.5 gm.) observed in period 26. There may have been some storage during periods 24 and 25. We suspect also that under these conditions there has probably been

TABLE 3-*a*

Weight and Nitrogen Balance

Dog 33-11.

Period 7 days	Diet	Weight	Nitrogen balance: Intake: In diet	Intake: In excess R.B.C. injected	Output: In plasma	Output: In urine	Output: In feces	Intake minus output
		kg.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
19	Liver basal (hemolysis)	11.9	17.5	12.0	8.7	18.1	3.0	−0.3
20	Liver basal	11.8	17.5	1.8	7.1	13.9	2.6	−4.3
21	Liver basal	11.8	16.6	6.3	5.1	13.8	2.2	1.8
22*	Liver basal	11.8	17.5	6.4	4.4	13.8	2.7	3.0
23	Liver basal + tryptophane, 4.2 gm.	11.5	18.1	1.3	3.5	11.6	2.9	1.4
24	Liver basal	11.6	17.5	2.6	2.9	11.7	3.1	2.4
25	Liver basal	11.8	17.5	1.2	3.2	12.1	2.4	1.0
26	Fasting + abscesses	10.7	0.0	4.1	2.5	11.6	1.8†	−11.8
27	Liver basal	11.1	17.2	2.7	2.7	9.9	3.2	4.1
28	Liver basal	11.3	17.4	3.0	3.9	10.0	3.1	3.4
29	Liver basal	11.6	17.5	1.1	3.2	10.0	3.1	2.3
30	Liver basal	11.5	17.1	−0.1	2.6	10.3	4.3	−0.2
Totals....			191.4	42.4	49.8	146.8	34.4	2.8

* 8 day period recalculated on basis of 7 days.

† Nitrogen in pus from abscesses, fecal nitrogen included in following period.

some *conservation* of the split products produced by the abscesses with production of new plasma protein. There is little or no rise in urinary nitrogen which is a very different response from the reaction with abscesses in the undepleted dog.

Red cell hematocrits in this experiment (Tables 2 and 3) were kept at a level above the initial level for this dog of 47 per cent. It is possible that this favored red cell destruction and removal by the

normal mechanism and accounts in part for the heavy intake values for red cell nitrogen. The figures for this dog are about 3 times as high as for dog 36-95 (Table 4-*a*) which received about 1 gm. of nitrogen a week from excess red cells injected.

Clinical Experimental History.—Dog 33-11 (Tables 2 and 2-*a*; 3 and 3-*a*). An adult female mongrel, born Nov. 25, 1932, this dog has been the subject of two periods of plasmapheresis previously reported (7, 5). Between the end of the latter period and the beginning of the present observations 20 weeks elapsed. During the 1st week (Tables 1 and 1-*a*) the dextrose was given by stomach tube. The *kidney basal* ration contained 50 gm. (raw weight) cooked pork kidney; canned tomato, 25 gm.; Vitavose (Squibb), 5 gm.; cane sugar, 95 gm.; butter fat, 10 gm.; lard, 30 gm.; cod liver oil, 10 gm.; bone ash, 5 gm.; salt mixture, 1 gm.; and furnished 9.15 gm. protein and 908 calories. By mistake one day's diet was omitted in the 4th week.

The *liver basal* diet contained liver, 75 gm.; canned tomato, 50 gm.; cane sugar, 100 gm.; lard, 30 gm.; cod liver oil, 15 gm.; bone ash, 20 gm.; salt mixture, 1 gm.; and furnished 15.6 gm. protein and 920 calories. About 5 per cent of this diet was vomited one day during the 1st week it was given. When gelatin 15 gm. was added to this ration in the 11th and the 16th weeks the cane sugar was reduced by 14 gm. to maintain a constant daily caloric intake. The granular gelatin was mixed into the basal diet without addition of water. The "protein-free" diet (calculated for a 11.7 kilo dog) contained cane sugar, 102.8 gm.; dextrin, 33.9 gm.; lard, 31.1 gm.; butter, 12.2 gm.; bone ash, 4.4 gm.; salt mixture, 2.2 gm., giving a total of 920 calories. The vitamin supplements given in conjunction were the same as those chosen by the original users of this diet (8): 4 tablets of cod liver oil concentrate and 8.2 cc. of the liver extract-rice polishing extract suspension. This diet was not well taken and small regurgitations during the 7 days amounted to a total of 80 per cent of a single day's diet. In the 16th period, *l*-tryptophane was added, 0.1 gm. per day for the first 3 days and 0.6 gm. per day for the last 4 days.

On the 2nd day of the 19th period the 136 cc. donor red cells given to replace the 119 cc. cells withdrawn from the dog in the day's bleeding were evidently injured by heat. The hematocrit dropped and the plasma was red with hemoglobin the next day; hemoglobinuria occurred and the animal was clinically upset. On the 5th day of the period the animal appeared largely recovered, the urine was pale yellow, but the plasma was slightly icteric. By the 7th day the plasma was clear. Throughout the entire episode the animal ate all of its diet. In the 21st period 30 per cent of one day's diet was vomited for no apparent reason. The dog was mildly upset in the first days of the 23rd period: ate all of its food but with less speed, and had slight diarrhea, probably slight edema. The latter could be demonstrated only by firm pressure of the tissue about the Achilles tendons. The plasma protein concentration on the last day of the 22nd period

reached the low point for the entire course of this experiment of 3.56 gm. per 100 cc.

For production of acute *sterile inflammation*, turpentine, 0.8 cc. was injected subcutaneously on the left side on the last day of the 25th period. The following day the rectal temperature was 39.7°C. and the leucocyte count 42,200. The next day the temperature was 37.4°C. but the leucocytosis 62,800. The following day (3rd of the 26th period) a fluctuant *abscess* was present on the left side, and turpentine 0.8 cc. was injected on the right. Next day temperature was 39.4°C. and leucocyte count 40,600. Both abscesses were drained 3 days later (7th day of 26th period), the left yielding 35 cc. and the right 100 cc. of blood-stained pus. Leucocytosis persisted (to 60,000) in the first of the following (27th period) but had dropped to 13,200 by the end of this period. The right abscess cavity drained another 45 cc. pus during this time. Pitting edema of moderate degree appeared in neck and legs at the beginning of this 27th period, despite the high plasma protein concentration (note low A/G ratio). The edema gradually disappeared and was scarcely perceptible in the 28th period.

Tables 4 and 4-*a* show that the reserve of plasma protein building material was depleted by one week of fasting plus 2 weeks of depletion on the liver basal diet. This reserve protein store in this dog amounted to 25 gm. plus the amount used from this store during the fasting week. About 7.0 gm. is accounted for by the drop in plasma protein concentration from 5.25 to 4.02 gm. per cent. The dog gained weight on this liver basal in spite of a week's fast and 2 weeks of protein-free diet.

Protein-free diet (periods 6 and 7) gives valuable information and shows clearly that when the protein reserve store is exhausted the dog can produce very little plasma protein indeed. The first protein-free week (period 6) shows an output of 8.4 gm. as compared with 3.4 gm. protein output for the 2nd week (period 7). This *surplus* of 5 gm. plasma protein in period 6 probably relates to a "carry over" from the preceding week of liver basal diet and represents materials from that diet in "process of production" from the food amino acids to the finished product (plasma protein). During the 2nd week of protein-free diet the dog could furnish to the plasma only 3.4 gm. protein. The plasma protein level was kept very constant and there was the usual fasting *shrinkage* of the blood *plasma volume* which would account for 1.4 gm. of the plasma protein removed. This brings the actual protein produced in period 7 to a mere 2 gm. which indicates how difficult the body finds its task of producing plasma protein when

all reserves are exhausted and only traces of "protein" are coming in through the digestive tract.

On the first day of the 8th period (Table 4) 24 hours after the resumption of the liver basal the circulating plasma protein concentration had jumped from 4.06 per cent, on the last day of the 7th period,

TABLE 4

Blood Plasma Protein Production as Influenced by Protein-Free Periods and by Interruption of Plasmapheresis

Dog 36-95.

Period 7 days	Diet	Protein intake Total for 7 days	Plasma protein removed Total for 7 days	Protein removed above basal*	Blood plasma Average concentration		R.B.C. hematocrit, average	Plasma volume
					Total protein	A/G ratio		
		gm.	gm.	gm.	per cent		per cent	cc.
	Kennel				5.34		47.4	603
1	Dextrose, 350 gm.	0	0.8		5.25	1.4	50.5	486
2	Liver basal	111	38.6		4.63	1.3	51.8	524
3	Liver basal	111	25.1	24.9	4.04	1.2	49.6	546
4	Liver basal	111	20.3		4.02	1.1	48.6	507
5†	Liver basal	111	20.1		4.04	1.1	48.8	533
6‡	Protein-free	4	8.4		4.03	1.1	51.4	526
7	Protein-free	4	3.4		4.00	1.0	50.8	489
8	Liver basal	111	25.1		4.22	0.9	49.0	526
9§	Liver basal	111	3.9		4.45	1.0	46.3	524
10§	Liver basal	111	4.0		4.56	1.2	45.3	570
11	Liver basal	111	28.4		4.33	1.1	50.1	546
12	Liver basal	111	31.8		4.26	0.9	51.8	565
13	Liver basal	111	29.8	−2.1	4.12	0.9	50.2	567

* Estimated basal output on liver diet = 20 gm. plasma protein per week.
† 9 day period recalculated on basis of 7 days.
‡ 5 day period recalculated on basis of 7 days.
§ No plasmapheresis.

to 4.45 per cent, a quick response to the ingestion of protein. Plasmapheresis was over-vigorously employed so that by the first day of the 9th period the circulating protein level had been reduced to 3.87 per cent. This explains in part at least the slightly greater plasma protein output in period 8.

Plasmapheresis was *interrupted* completely during periods 9 and 10

(Table 4). The plasma protein removed represents plasma withdrawal associated with the necessary sampling and blood volume determinations. During the 5 weeks (periods 9 to 13) in Tables 4 and 4-*a* the basal diet was continued and the *expected* plasma protein output was 100 gm. *Actually* during the last 3 weeks 97.9 gm. were re-

TABLE 4-*a*

Weight and Nitrogen Balance

Dog 36-95.

Period 7 days	Diet	Weight	Nitrogen balance: Intake, in diet	Intake, in excess R.B.C. injected	Output, in plasma	Output, in urine	Output, in feces	Intake minus output
		kg.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
	Kennel	13.1						
1	Dextrose, 350 gm.	11.7	0.0	−1.0	0.1	12.8	0.7	−14.6
2	Liver basal	11.8	17.8	0.4	6.3	9.7	8.3*	−6.1
3	Liver basal	11.8	17.8	1.0	4.1	12.6	4.2	−2.1
4	Liver basal	12.2	17.8	1.0	3.4	11.1	3.8	0.5
5†	Liver basal	12.2	22.9	3.3	4.3	15.3	4.2	2.4
6‡	Protein-free	12.4	1.2	0.0	1.2	5.3	1.0	−6.3
7	Protein-free	12.2	1.6	−0.7	0.6	6.4	1.1	−7.2
8	Liver basal	12.3	17.8	1.4	4.1	10.5	2.9	1.7
9§	Liver basal	12.8	17.8	−2.2	0.6	8.7	3.2	3.1
10§	Liver basal	13.2	17.8	−0.4	0.6	9.1	3.2	4.5
11	Liver basal	13.5	17.8	6.1	4.5	10.0	2.6	6.8
12	Liver basal	13.3	17.8	0.5	5.2	12.7	4.0	−3.6
13	Liver basal	13.6	17.8	2.6	5.0	12.9	2.5	0.0
Totals........................			185.9	12.0	40.0	137.1	41.7	−20.9

* Includes some urinary nitrogen.

† 9 day period.

‡ 5 day period.

§ No plasmapheresis.

moved to return the dog to its true depletion level. This indicates clearly that during the 2 weeks of no plasmapheresis the dog stored 32 gm. plasma protein building material, while the plasma protein concentration did not rise above 4.75 per cent. Subsequently the dog withdrew from this store all of the material which was contributed to

the circulating plasma and removed in the last 3 weeks of plasmapheresis (periods 11 to 13). The maximum concentration of plasma protein during the period of no plasmapheresis was attained within 4 days after cessation of plasmapheresis and ample storage took place at or below this level. All of this emphasizes the ease with which the dog can shift protein from the circulating plasma to organs or tissues and subsequently reverse the process. More data on this mechanism have just been published (2, 4).

Clinical Experimental History.—Dog 36-95 (Tables 4 and 4-*a*). An adult male mongrel weighing 13.1 kg. was fasted for a week except for daily administration of 50 gm. dextrose by stomach tube. While completely consuming a daily diet of liver, 75 gm.; cane sugar, 50 gm.; corn starch, 50 gm.; lard, 25 gm.; cod liver oil, 25 gm.; canned tomatoes, 75 gm.; bone ash, 20 gm.; salt mixture, 1 gm.; kaolin, 15 gm., the *protein stores* were depleted by plasmapheresis. This diet was replaced in the 6th period by a "protein-free" diet (see Methods), calculated for a 12.2 kilo dog, and having the same caloric value, 960 calories, as the one described above. It contained cane sugar, 107.3 gm.; dextrin, 35.4 gm.; lard, 32.5 gm.; butter, 12.7 gm.; bone ash, 4.6 gm.; salt mixture, 2.3 gm.; and had the vitamin supplements of 4 tablets cod liver oil concentrate and 8.5 cc. rice polishings-liver extract suspension. This diet was readily consumed and retained, except for the vomiting of about 20 per cent of one day's intake in the 7th period. From the 8th through the 13th periods the liver diet described above was consumed completely. No plasmapheresis was performed during the 9th and 10th periods except for daily sampling for analysis and the return of washed red cells on one occasion in each period to maintain the hematocrit, 36 cc. and 60 cc. respectively.

DISCUSSION

Interesting experiments have been reported by Melnick and Cowgill (8, 9) which are obviously related to the problems involved in the experiments tabulated in this paper. A detailed discussion of the differences between their work and ours would not seem appropriate in this place. There are many differences in methods, experimental procedure, and research approach which are responsible for divergent opinions. We have no quarrel with divergent opinions as they usually stimulate work which eventually uncovers the truth.

Their belief (8) that a *single week* of rapid plasma depletion will exhaust the reserve store of plasma protein building material, we cannot share. Tables above and in other papers indicate our belief

that 2 to 6 weeks are required to exhaust this plasma protein reserve store and that dogs differ in this respect. These tables demonstrate that a plasma protein concentration of 4 per cent is usually reached and maintained for 1 to 4 weeks prior to complete exhaustion of the reserve store and is therefore no indicator of the exhaustion of this store. If this protein reserve is not exhausted the subsequent picture is confused as it relates to new protein production. We also believe that when the reserve protein store has been completely exhausted by plasmapheresis and continued low protein diet the dog can produce *very little new plasma protein* (2 to 4 gm. a week) on a "protein-free" diet. This small amount of new formed protein *may* be related to the wear and tear of tissue or organ protein and subsequent conservation of end products.

SUMMARY

When blood plasma proteins are depleted by bleeding, with return of the washed red cells (plasmapheresis) it is possible to bring dogs to a steady state of hypoproteinemia and a uniform plasma protein production on a basal low protein diet. These dogs are clinically normal with normal appetite, no anemia and normal nitrogen metabolism. These dogs become test subjects by which various factors relating to plasma protein production may be tested.

The normal dog (10 to 13 kg.) has a substantial reserve store of plasma protein building material (10 to 60+ gm.) which requires 2 to 6 weeks plasmapheresis for its complete removal. After this period the dog will produce uniform amounts of plasma protein each week on a fixed basal diet.

Dogs previously depleted by plasmapheresis and then permitted to return to normal during a long rest period of many weeks, may show much higher reserve stores of protein building material in subsequent periods of plasma depletion (see Table 1).

Under uniform conditions of low protein diet intake when plasmapheresis is discontinued for 2 weeks the plasma protein building material is *stored* quantitatively in the body and can subsequently be recovered (Table 4) in the next 2 to 3 weeks of plasmapheresis.

Given *complete* depletion of plasma protein building reserve stores the dog can produce very little (2± gm. per week) plasma protein on

a protein-free diet. This *may* be related to the wear and tear of body protein and conservation of these split products.

Abscesses produced in a depleted dog during a fast *may* cause some excess production of plasma protein which is probably related to products of tissue destruction conserved for protein anabolism.

Gelatin alone added to the basal diet causes very little plasma protein production but when supplemented by *tryptophane* gives a large protein output, while tryptophane alone is inert.

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UTERINE ADENOMATA IN THE RABBIT

I. Clinical History, Pathology and Preliminary Transplantation Experiments

By HARRY S. N. GREENE, M.D., and JOHN A. SAXTON, Jr., M.D.

(*From the Department of Animal and Plant Pathology of The Rockefeller Institute for Medical Research, Princeton, New Jersey*)

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In view of the widespread use of the rabbit in experimental laboratories and the scarcity of recorded instances of uterine adenoma and adenocarcinoma, it would appear that these tumors are of extremely low incidence. Polson (1) in a study of the incidence up to 1927 cited 29 instances, and a review of the subsequent literature yields only a few additional cases. It is of interest, therefore, that during the past 4 years in a colony of approximately 500 female rabbits more than 80 such tumors have been found.

Past reports have been limited to the pathological examination of tumors which were first observed at autopsy, and the clinical course has not been described. Attempts to transplant the tumor were made in the majority of the recorded instances but were uniformly unsuccessful. On the other hand, in many of the cases included in the present report, the presence of the tumor was noted during life and the clinical course followed to death or disposal. In addition, transplantation has been successful and growth has been obtained in the 6th series of transfers.

The object of this paper is to record the present high incidence of the tumor and to describe its clinical history, pathology and the results of preliminary transplantation experiments.

Materials and Methods

The material for the present report is based on 83 cases of adenoma and adenocarcinoma of the uterus observed between May, 1934, and December, 1937. In

the majority of cases the tumor was detected during life and its presence subsequently confirmed by laparotomy or autopsy. In addition, smaller and microscopic areas of early tumor development were observed during the routine postmortem examination of other animals. 61 of the tumor bearing animals died or were killed at various stages of tumor development, 8 died with metastases and 14 are still under observation.

The composition and management of the colony in which the tumors were found has been described in detail elsewhere (2).

The tumor was transferred to normal animals by the injection of cell emulsions and the transplantation of tissue fragments. Emulsions were made by grinding fresh tumor tissue with 0.85 per cent sterile sodium chloride solution and 0.3 cc. of the mixture was injected at the chosen site. Tissue fragments were selected from active appearing areas of the tumor and transplanted immediately.

Tumor material was transferred to the subcutaneous tissues, muscle, peritoneal cavity, testicle and anterior chamber of the eye. Intraocular transfers were carried out under a local anesthetic. The cornea was punctured at its upper margin with a corneal knife and a small amount of aqueous humor allowed to escape. An extremely small tissue fragment of less than 2 mm. in diameter was then inserted, using forceps with fine serrated points. The best results were obtained when the tissue was forced into the inferior angle of the iris; this was easily accomplished by applying pressure along the corneal surface with a blunt instrument.

Tissues for microscopic examination were fixed in Petrunkevitch's solution and stained with hematoxylin and eosin. Pituitary glands were fixed in Helly's solution; many were serially sectioned and a variety of stains was used in an attempt to differentiate the various cell types.

Clinical Course

The presence of tumor in the uterus of a living animal is not indicated by specific signs or symptoms, and detection of the growth is dependent upon physical examination. A systematic examination of all animals has been part of the routine conduct of this colony, and the majority of tumors have been detected as they became palpable. It is obvious, however, that the course of the growth is considerably advanced at the time of discovery. The histories of the 83 animals concerned in this report give no indication of the date of beginning neoplasia, but analysis shows that disturbances in reproductive function always precede detection of the tumor and constitute a definite phase of the disorder.

Preclinical Period.—The fertility and reproductive efficiency of the colony are under constant check, and it is significant that while the

early histories of affected animals were characterized by normal reproductive activity, a pronounced alteration in fertility, litter size and maternal care occurred during the period immediately preceding discovery of the tumor.

Fertility was greatly diminished for 4 to 5 months prior to detection of the growth. In one group containing approximately one-third of the animals, fertility ceased abruptly, but the last gestation period was normal in all respects and terminated in the birth of a litter of usual size which subsequently received good maternal care. In other animals, the percentage of sterile matings was greatly increased, but occasional pregnancies resulted, and in 7 instances matings made within 2 months of tumor detection proved fertile. The litters born to animals of this group were reduced in size, contained many dead young and desertion by the mother was common. The reduction in litter size was frequently abrupt with a diminution to less than one-half of the average number of young; in other instances the reduction was gradual and became more pronounced as the date of tumor detection was approached.

Other abnormalities of gestation were common in animals of this group. There were 3 instances in which whole litters were retained *in utero* far beyond the limits of normal gestation and 4 instances of abdominal pregnancy. In addition, there were 21 cases in which the products of conception were either aborted or resorbed during the 2nd week of gestation. The usual history in such cases was that pregnancy was diagnosed 10 days after a mating on the basis of uterine nodules resembling pregnancy cysts, but delivery failed to occur at the end of an average gestation period, and subsequent examination revealed an empty uterus.

In general the period of altered reproductive function extended for 4 to 6 months prior to the clinical detection of the tumor. No consistent changes were noted on palpation of the uterus during this period, but in view of the persistent occurrence of reproductive abnormalities, it is reasonable to assume the presence of functional alterations. Moreover, inasmuch as the postmortem discovery of extremely small tumor nodules not palpable during life, was also preceded by long periods of reproductive disturbances, it seems probable that the functional alteration may have been present before the initiation of neoplastic changes.

Period of Growth.—

Detection of the tumor depends on the palpation of uterine nodules which persist and increase in size. The nodules when first noted, approximate the size of 10 day pregnancy cysts. They are frequently multiple and may be present in

both uterine horns. In such cases their distribution also resembles that of pregnancy cysts. The differential diagnosis rests on the firmness, absence of fluctuation and slow growth of the tumor nodules.

The rate of growth varies in different animals. The tumors may reach the size of hens' eggs in 6 months, or may remain barely palpable for a similar period. Occasionally, large tumor masses have been found to regress in size, and it is a point of interest that such an occurrence has been repeatedly observed prior to metastasis. In other instances metastasis has occurred without a palpable increase in the size of the primary nodules, which have remained no larger than 1.5 cm. in diameter for more than a year.

In approximately one-quarter of the cases cystic breast changes were noted when the uterine tumor was first detected. The changes were either diffuse or limited to a single breast, and on microscopic examination were similar to those found in chronic cystic disease in man. Subsequently, definite tumors developed in the affected breasts of 3 animals. The growth in one instance was a pure adenoma in type while the tissue changes in the others resembled those of a fibroadenoma. In one of the latter cases the breast tumor progressed more rapidly than the uterine and the animal eventually died with metastases from that growth.

The fertility of tumor bearing animals has been tested in numerous instances and all matings have proved sterile. Sterility is apparently not due to the obstruction caused by the tumor mass, for repeated matings of animals with small nodules limited to one horn have also been uniformly infertile.

There are no constant manifestations in the general behavior of an animal other than those associated with breeding activity to indicate the presence of tumor. Frequently, during the course of the tumor, animals exhibit signs generally associated with pregnancy. The breasts become engorged, the temperamental changes common to the end of gestation appear and the animals pull fur and build nests. Similar manifestations have been observed in normal animals during pseudopregnancy resulting from a sterile mating, but the animals in question had not been mated for long periods, and it is assumed that an endocrine disturbance was associated with the presence of the tumor.

There is no evidence that the tumors are painful and the uterus may be palpated without apparent tenderness. Bleeding is a rare

manifestation and occurs only in late stages. Cachexia does not occur as long as the tumor is confined to the uterus.

Termination.—

Many tumor bearing animals have been killed for pathological examination, but others have been held to determine the ultimate fate of the growth. The clinical course has ended in death with metastases in 8 instances. The duration of the disease in these animals as computed from the date of discovery of the tumor varied from 5 to 20 months and averaged 12 months, but if determined from the beginning of the period of reproductive disturbance, the course varied from 14 to 29 months and averaged 19 months.

The period of observation of other affected animals has not exceeded 9 months, and it is significant that metastasis has occurred in all cases in which the known duration of the tumor has been greater than 1 year.

In 4 instances tumor bearing animals have died of a disorder that was clinically identical with toxemia of pregnancy (3). Postmortem examination revealed lesions typical of that condition, but in all cases the uterus was empty save for the tumor masses, and microscopic examination gave no evidence of the cellular changes characteristic of pregnancy or pseudopregnancy.

Incidence

A detailed analysis of the incidence of the uterine tumor is beyond the scope of this paper, but certain facts are worthy of note in the present connection.

The frequency of cases during the past 6 years is shown in Table I. The abrupt increase in 1936 cannot be attributed to changes in material or methods. The female population has not increased, but has been maintained at a constant level of from 400 to 500 animals throughout the period. Moreover, the composition of the population has not changed and there has been no alteration in the routine conduct of the colony.

Age.—There have been no instances of uterine tumor in animals less than 2 years of age. The tumors have been found during the 3rd year of life in 20 per cent of the cases, during the 4th year in 44 per cent, the 5th year in 25 per cent, the 6th year in 9 per cent and the 7th year in only 1 per cent of cases. The average age of animals at discovery of the tumor has been 45 months.

Parity.—The incidence of the tumor has so far been limited to multiparous animals, but it should be noted that there have been no virgin females and relatively few primiparae over 2 years of age in the colony. Previous to clinical recognition of the disease, the affected animals had borne from 2 to 16 litters over a period varying from 12 to 75 months. The average number of litters was 7 and the average breeding period 38 months.

Breed and Family.—The tumors have been found in animals of Beveren, Dutch, English, Himalayan, Havana, Marten, Polish, Sable, Tan and Rex breed, but not in members of the Belgian, Chinchilla or Silver breeds. Many of the tumor bearing animals were closely related, and instances of the disease in 3 generations of a family are exceedingly common. The frequency of the tumor is highest in the same breeds and lines of animals in which the incidence of toxemia of pregnancy is greatest (4).

TABLE I

The Incidence of Uterine Tumors from 1932 to 1938

Year	Number of cases
1932	0
1933	0
1934	3
1935	1
1936	17
1937	62

Pathology

The tumors arise from the glandular epithelium of the mucous membrane of the uterus and preserve an adenomatous structure throughout their course. The gross and histological characteristics and the changes noted at various stages of growth have been similar in all animals, and there is no question of the identity of the pathological process in different cases.

The Primary Tumor

Tumors of the uterus cannot be detected in living animals until the mass has reached a diameter of approximately 1 cm., but routine postmortem examination

has revealed the presence of smaller growths in a number of instances. The early growths are usually pedunculated and vary from 0.2 cm. in diameter (Fig. 1). Extremely small nodular thickenings of the endometrium have also been found which histologically show the characteristic structure of the tumor (Fig. 2).

At autopsy of the earliest clinically recognized cases the tumors are polypoid and are usually situated on the mucosal folds adjacent to the mesometrial insertion. They present a smooth glistening surface and are obviously covered by intact lining epithelium. In the majority of cases the tumors are multiple and may be regularly spaced throughout both horns. In such instances the different masses closely approximate each other in size and appearance. Solitary tumors have been found in all parts of the uterus, but occur with the greatest frequency in the segment adjacent to the Fallopian tube. The right and left horns are involved with equal frequency.

Older tumors are usually sessile, but extend deeply into the myometrium and are visible through the peritoneal covering. Occasionally, the preponderance of growth is directed into the uterine lumen, giving rise to sausage shaped masses attached by narrow bases and filling the greater part of one horn. Single tumors may attain a diameter of 4 cm.; the larger masses noted clinically are, as a rule, made up of 3 or more distinct tumors arising from different parts of the uterine circumference and forced into triangular shape by appositional growth.

At this stage tumors may be found in all portions of the mucosa (Fig. 3), and frequently small nodules occupy a position diametrically opposite larger masses, suggesting an implantation origin. The surfaces of older growths show deep ulcers with irregular edges and necrotic bases, and large areas of necrosis appear on section.

In advanced cases the endometrium and the uterine muscle may be almost entirely replaced by neoplastic nodules. Metastasis is often associated with an extreme degree of necrosis of the intrauterine tumor, and the greater part of the mass may slough and be discharged through the vagina. As a result, clinical examination at this stage shows a marked diminution in the amount of palpable tumor.

The histological structure of the tumor varies in different stages of development, but in general is characterized by an atypical arrangement of glandular elements in a variable stroma.

Early changes which may be interpreted as the beginning of the neoplastic process, have been observed in uteri showing no gross tumor (Fig. 4). In such cases small groups of atypical alveoli lie directly beneath the surface epithelium in direct continuity with normal appearing endometrium. The alveoli are irregular in size and often show branching processes. The lining epithelium is generally flattened, pale staining and contains scattered mitotic figures. A slight lymphocytic invasion and the formation of new connective tissue cells usually accompanies such changes.

The two grossly different types of tumor noted in early cases show histological variations which suggest differences in growth rate. The pedunculated

masses are composed of irregularly scattered atypical alveoli embedded in an abundant myxoid stroma rich in blood vessels (Fig. 5). Many alveoli are lined by columnar cells arranged in multiple layers, while in others the cells are low and flattened. Occasionally, both types of epithelium are found in different parts of the same gland. Throughout the greater part of the mass, alveoli are separated by large amounts of stroma, but in scattered areas they tend to be collected in more or less compact groups. This tendency is most marked close to the base of growth which rests directly on the uterine musculature.

The nodular endometrial thickenings on the other hand, display characteristics of more active growth (Fig. 6). Stroma is scanty and the nodule consists for the most part of epithelial elements. The structure is compact and individual alveoli are separated by a minimum of stroma. About the periphery toward the uterine lumen the alveoli are dilated and lined by a single layer of epithelium, but adjacent to the muscle, coiled branching processes and papillary proliferations are so numerous that they form almost solid cell groups. The underlying muscle is distorted and separated from the tumors by a compressed connective tissue capsule. Growth is apparently expansive rather than invasive at this stage of development.

Clinically palpable tumors usually show extensive invasion of the muscular coat (Fig. 7) and structural changes indicative of advanced anaplastic growth. Well formed alveoli are present in scattered areas, particularly toward the center of the mass, but in the greater part of the tumor the arrangement of epithelial cells bears little resemblance to normal glandular architecture. Many alveoli are large, dilated and irregular in shape with multiple branching processes. In others, papillary epithelial proliferations tend to obliterate the lumen or interlacing bands of supporting stroma form secondary alveoli.

Active proliferative changes are most marked in the advancing edge of the growth and in isolated intramuscular nodules. In these situations individual cells are irregular in size, but are generally columnar in shape with large vesicular nuclei and occasional mitotic figures. On the other hand, epithelial cells lining alveoli in the peripheral zone toward the uterine lumen tend to be flattened with small hypochromatic nuclei and pale eosinophilic cytoplasm. Both types, however, may be interspersed in any selected area.

More advanced stages are characterized by deep muscular invasion and extension laterally in the endometrium. In some instances, the advancing edge of the growth may appear as a solid sheet or the cells may assume a spindle shape; but in general there is no further dedifferentiation of epithelium and the cells are similar in morphology and arrangement to those described in the previous paragraph. Necrosis is common in the center of the mass and along its free margin. The surface epithelium is usually absent over peripheral necrotic areas and as a result, tumor tissue lies in direct contact with the surrounding endometrium. Sections from the opposing uterine wall often show small areas deeply infiltrated with tumor cells which were presumably implanted from the ulcerated primary growth.

It should be emphasized that in the majority of cases the neoplastic change occurs in multiple foci, giving rise to multiple primary nodules which progress at the same rate and show identical histological changes at different stages of development. Rarely, the various centers of growth remain discrete throughout their course, but usually coalesce by lateral extension, and with deep muscular invasion and growth of secondary nodules, the entire uterus is converted into a mass of tumor tissue.

Metastases

Invasion of vascular walls and rupture into blood vessels is frequently observed in histological sections obtained from tumors in early clinical stages (Fig. 8), but metastatic growths have only been found late in the course of the disease and are always associated with extensive necrosis of the primary tumors.

Gross and microscopic metastases have been found in all organs of the abdomen and thorax, in the thyroid gland and in the femoral bone marrow. The peritoneum, pleura and pericardium are commonly involved, and nodules occur frequently in the walls of the stomach and intestine.

In one instance a solitary mass the size of a golf ball was found embedded in the wall of the duodenum, but in other cases metastases have been multiple and widespread. Variations in size and appearance suggestive of considerable age difference are common. As a rule, nodules on serous surfaces are small and miliary in appearance while large necrotic masses are found in parenchymatous organs.

Dissemination also occurred by direct extension of the primary growth into the mesometrium and surrounding structures. The rectum, vagina, bladder and pelvic lymph nodes may be diffusely infiltrated or the growth may appear as isolated nodules in these situations. Further spread anteriorly through the lumbar fat is common, and in one instance the growth extended continuously on both sides from the uterus to the diaphragm with involvement of the mesentery, omentum and all abdominal organs.

Metastatic nodules in general preserve the glandular structure of the primary tumor (Fig. 9), but occasionally exhibit a more cellular, less organized growth. In some instances the influence of changed

environment on tumor structure was particularly prominent. Thus, in one animal, the lung metastases bore a marked resemblance to squamous cell carcinoma while in other locations the arrangement of cells resembled an endothelioma. In another instance, the stroma of metastatic nodules was hyperplastic and suggestive of sarcomatous proliferation.

Changes in Other Organs

Pathological changes rarely found in other conditions have been observed with high frequency in the thyroid and suprarenal glands of tumor bearing animals, but other organs show no consistent alteration.

The suprarenals and thyroid are not altered in the gross, but histological changes are present in all stages of the disease. The greater part of the thyroid is composed of closely packed, small, round acini lined by low cuboidal epithelium (Fig. 10). Many alveoli contain a small amount of deeply eosinophilic colloid, but in others a lumen is lacking. No normal alveoli are present and the structure closely resembles that characteristic of fetal adenomata in man. In other portions of the gland single alveoli may be widely dilated with the production of macroscopic cyts, while contiguous areas contain smaller irregularly dilated alveoli, many of which intercommunicate through degenerated septal walls. The cystic areas are lined by flattened epithelium and contain pale staining structureless material.

The changes in the suprarenal are most marked at the inner boundary of the zona fasciculata and resemble those observed in toxemia of pregnancy in the rabbit (Fig. 11). The cells of this area contain large amounts of lipoid-like material and often coalesce to form rounded masses. The entire inner circumference is frequently involved and the process so pronounced that the cortex and medulla are separated by a wide zone of more or less homogeneous pale staining material interspersed with nuclei in various stages of degeneration.

The histological appearance of the hypophysis is not consistent. In many animals, sections of the anterior lobe stained with Mann's hematoxylin and eosin show an increase in non-granular cells, but whether such cells are chromophobes or depleted chromophile types has not been determined. The intermediate lobe is usually wide and contains an abundance of colloid. Similar colloid material is also found in scattered areas throughout the anterior lobe.

The ovaries may or may not contain corpora lutea, depending upon recent mating history. There has been no evidence of spontaneous production of corpora lutea in any of the animals examined. When corpora lutea are present, however, there are associated changes in the uterus of a decidual nature, such as glycogen cells of the endometrium, and also syncyctial epithelial giant cells. Minot's monster cells have also been observed in a number of such cases and they are

probably remnants of fetal elements following spontaneous abortions. Changes in the endometrium preceding or associated with the development of these tumors appear to inhibit proper development of the fetus, leading either to abortion, or fetal resorption. Abortion is difficult to detect in the rabbit.

Transplantation

Autotransplantation.—Tumor tissue obtained from the uterus by biopsy has been transferred to the subcutaneous tissues of various parts of the body in numerous instances and growth has occurred in all cases. The resulting tumors grow to a large size and may invade underlying muscle, but extension to the skin has not been noted. Histologically, the epithelial elements of the transplant are similar to those of the primary tumor, but in many instances the stroma is hyperplastic and contains areas suggestive of sarcomatous proliferation.

Homotransplantation.—Various methods of transplantation have been employed in an attempt to transfer the tumor from affected to normal animals. Intratesticular, subcutaneous, intramuscular and intraperitoneal inoculations have not been successful. Transplantation into the anterior chamber of the eye has given positive results, however, and intratesticular inoculation has been successfully carried out with tissue derived from an eye transplant following the 5th serial transfer.[1]

Intraocular growth has been obtained both from the injection of cell emulsions and from the transplantation of tissue fragments. Transfer of the spontaneous tumor by means of cellular emulsions has been successful in one out of four attempts, while the use of tissue fragments has resulted in 50 per cent of takes in the same number of trials.

Successful transplantation has been effected by using tissue derived from primary uterine tumors as well as from metastatic growths. The transfer of growing tissue from tumors entirely confined to the uterus has been accomplished in only one out of five trials. In this single instance, the transplanted fragment persisted without appreciable change for a period of 4 months. Slow growth was noted during

[1] Heterotransplantation experiments are in progress, and to the present time progressive growth of the transplant has been obtained in the eye of the guinea pig, using tumor material derived from the 6th serial eye transfer in the rabbit.

the following 3 months and the nodule increased to approximately 0.25 cm. in diameter. Thereafter, the tumor decreased in size and 9 months after transplantation, had completely regressed.

On the other hand, progressive growth followed the transplantation of tissue derived from the primary tumors of 2 animals that showed widespread metastases. The incubation period in both instances was less than 2 months in duration and the fragments grew to large size. Portions of the fragments were later removed and transplanted into the eyes of other animals. Active growth resulted, and in one instance the tumor was successfully transferred for 3 generations by serial transplantation.

The single attempt to transplant a metastatic nodule was also successful, and at the present time this tumor has been carried through 6 generations of transfers. Growth was apparent in the majority of animals 1 month from the date of the first transplantation, but in subsequent generations the period of incubation decreased and takes could be recognized 2 weeks after the 6th transfer. The proportion of successful transplants increased in each generation, and the 6th transfer resulted in 100 per cent of takes.

The sequence of events in the transplanted tissue can be easily followed, particularly in the unpigmented eyes of albino and Himalayan animals (Fig. 12). Usually, the first sign of growth is a slight reddening of the tissue, and frequently a thin web-like membrane may be seen growing from its periphery. Unsuccessful transplants are opaque and greyish white in color. The time of vascularization is variable and is apparently dependent upon the position of the transplant with relation to the angle of the iris. The growth rate is increased after vascularization, and in less than a month the anterior chamber may be completely obliterated by rapidly growing tumor tissue.

Following the 4th and subsequent transfers, it was noted that growth frequently occurred in multiple foci in a line between the corneal incision and the transplanted fragment, appearing first as pin point milky spots which gradually increased in size and eventually coalesced to form a nodule as large as that of the tumor fragment. It is assumed that the accessory growths arise from cells or minute tissue fragments dislocated from the original graft during its passage through the anterior chamber. Histologically, the transplants at this stage show an increased cellularity and a less organized structure which may account for the greater fragility (Figs. 13 and 14).

There has been no evidence that the frequency of takes in the first transplantation is influenced by the degree of relationship be-

tween tumor bearing animals and animals used for transfer. Moreover, although relatively few animals have been tested, there has been no indication that the susceptibility to transplantation varies with breed or genetic constitution. In early transfers the proportion of takes and the rate of growth was greater in females than in males, but in later transfers no difference was noted.

The length of the period of observation has not been adequate for determination of the eventual fate of the transplanted tumor. Metastasis has not occurred, but none of the transplanted growths have been under observation for a period of time equivalent to the duration of the course of spontaneous tumors before metastasis. In 2 instances the tumor has completely regressed after filling the greater part of the anterior chamber, but in other animals the growth has invaded the cornea and extended deep into the eye.

A portion of the growing tumor has been removed, in many cases, and transferred elsewhere in the body or transplanted to different sites in other animals. After operation there is usually a sudden increase in the growth rate of the remaining intraocular tumor, but surgical interference is occasionally followed by complete regression of the growth. To date reinoculation of the tumor has resulted in 2 subcutaneous growths, and transfer to the testicle of a different animal by means of cellular emulsions has been successful in 3 instances. Serial intratesticular inoculation with tumor material from the testicle of one of these animals has been carried to the 3rd generation at the present time (Figs. 15, 16 and 17).

DISCUSSION

The frequent occurrence of an adenomatous endometrial tumor in a colony of rabbits has afforded an unusual opportunity for the detailed study of many cases. The uniformity of essential characteristics displayed in different animals establishes the tumor as a distinct pathological entity, and a consideration of its salient features suggests a close analogy with adenocarcinomata of the corpus uteri in women.

The similarity of the clinical course and histological appearance of the two tumors is outstanding. Both tumors arise from the glandular epithelium of the uterine mucosa and are characterized by an atypical alveolar structure. A multicentric origin is usual in

the rabbit and probably also occurs in the more common diffuse variety of adenocarcinoma in women. The tendency toward early muscular invasion and late extrauterine extension shown by the human tumor is paralleled in the rabbit, and an eventual termination with metastases or diffuse local spread is common to both species.

Many animal tumors display special characteristics and analogous counterparts do not occur in human pathology. The high incidence of this tumor and the ability to transplant the growth are, therefore, of importance, not only as an additional source of material for investigation of the general problems of neoplasia, but also as they supply a means for experimental study of a particular type of growth that has its counterpart in a relatively common carcinoma of women.

The sudden increase in the incidence of cases of uterine tumor that occurred in 1936 is suggestive of an epidemic phase of an infectious disease. It should be pointed out, however, that the recent abrupt increase in the incidence of toxemia of pregnancy also simulated an epidemic (4), and epidemic-like outbreaks of other non-infectious disorders have been repeatedly observed in the colony in question. Filtration experiments have not been performed, but the absence of any sign of bacterial infection indicates that if the disease is of infectious origin, the agent is probably a filterable virus. However, there has been no evidence of contagion. Cases of the tumor are distributed irregularly throughout the colony and the incidence is not increased in the vicinity of affected animals.

The constant occurrence of multiple lesions in tumor bearing animals is evidence of a systemic disturbance. The disease is not confined to the uterus, and the presence of tumor in this organ is apparently only a local manifestation of a constitutional disorder. The suprarenal and thyroid glands show distinctive changes even in the earliest stages of tumor development. The hypophysis is also altered and the mammary glands frequently show productive changes.

Alterations of the suprarenal identical with those found in tumor bearing animals have been noted in mice after treatment with estrogenic substances (5), and there have been numerous reports of comparable breast changes following similar treatment. Moreover, a degranulation of chromophiles and an increase in the number of chromophobes have been found in the hypophyses of rats (6) and mice (7) after the continued administration of estrone, and similar

changes were noted in the hypophyses of a number of tumor bearing animals. In addition, in one reported case a mouse subjected to this treatment showed an adenoma of the thyroid, together with alterations in the suprarenal, hypophysis and breast (8).

The same organs affected in mice treated with estrone are altered in tumor bearing rabbits. Moreover, the pathological changes are similar in the two animals. Such conformity indicates that the exciting agent may be of a like nature in both instances, and in view of the known carcinogenetic action of estrone, it is not unreasonable to suggest that the tumors may also arise on the same basis and represent a natural analogue to the experimental production of neoplasia with this substance.

More direct evidence in regard to the status of estrone in tumor bearing animals is being sought, and while biological assay of tumor material has so far been made in only a single case, the results were positive in this instance and showed the presence of estrogenic substances.

The clinical histories of tumor bearing animals are characterized by long periods of infertility and reproductive abnormality which antedate the discovery of tumor and suggest a long continued endocrine disturbance. In many cases no uterine abnormality was found to account for the reproductive disorders, but in other instances cystic and hypertrophic endometrial changes accompanied the tumors at an early state and they may have been present for a longer period. Similar alterations of the mucosa have been repeatedly observed in experimental animals following the administration of estrone, and occur naturally in conditions associated with an abnormal production of estrogenic substances.

It is difficult to account for the recent high incidence of the tumor, particularly if it is of endogenous origin and based on an endocrine abnormality. The sudden increase of cases began simultaneously with an outbreak of toxemia of pregnancy; the highest incidence of both disorders is found in the same breeds and genetic lines, and occasionally tumor bearing animals die with the characteristic lesions of toxemia. Clinical and pathological investigations indicate that toxemia of pregnancy is also of endocrine origin, and while the two disorders are probably related, the relationship has not as yet been clarified and at the present time there is no satisfactory explanation for the "epidemic" occurrence.

A point of interest in regard to metastasis of the tumor should be emphasized. It was noted that invasion of vascular walls and rupture into blood vessels occurred during early stages of growth, but that metastasis was delayed until late in the course of the tumor, usually after the lapse of a year or more. It must be assumed that tumor cells enter the blood stream following the early rupture of vessel walls and either perish or fail to proliferate in distant parts. Microscopically, the appearance and arrangement of the cells of the primary growth at the time of metastasis are not greatly altered and show no more anaplastic tendencies than were observed at earlier periods. The opportunity for dissemination of cells is present long before metastasis actually occurs, and the successful transplantation of tissue from both metastasizing and non-metastasizing tumors to the anterior chamber of the eye is evidence that the ability to proliferate in a changed environment is not limited to the cells of the former growth. It seems improbable, therefore, that the production of metastatic growths is dependent solely upon the release of neoplastic cells into the blood stream.

A detailed examination of the tissues of animals following metastasis shows no consistent alteration from their previous condition. The only constant finding that differentiates non-metastasizing from metastasizing tumors is the presence of large areas of necrosis in the latter. This finding assumes significance when considered in connection with experiments performed in this laboratory (9). Casey, working with the Brown-Pearce tumor showed that necrotic tumor material possessed an enhancing principle and that the absorption of this material resulted in an increase in tumor growth and in the frequency and number of metastases. It is well recognized in human pathology that necrotic areas frequently occur in malignant tumors, but it is generally assumed that they result from the failure of stroma and blood supply to keep up with the rapidly increasing epithelial growth, and thus follow rather than precede malignancy. In the present instance, however, it is certain that necrosis precedes metastasis, and it is not improbable that the absorption of necrotic tumor material plays an important part in the subsequent history.

SUMMARY

83 cases of an adenomatous tumor of the uterine mucosa have been observed in a colony of rabbits during the past 4 years The results of a clinical and pathological study of the tumor, together with a description of transplantation experiments are included in the present report.

The clinical histories of tumor bearing animals are similar in all cases. Discovery of the tumor is preceded by a long period of reproductive disturbance, and its subsequent course is one of slow, continuous growth which has terminated in death with metastasis in all animals held under observation for longer than 1 year.

Microscopically, the tumor shows an atypical alveolar structure, and its characteristics closely resemble those of an adenocarcinoma of the uterine fundus in women. Pathological changes similar to those observed in mice after treatment with estrogenic substances occur in the thyroid, suprarenal, pituitary and mammary glands.

Intraocular transplantation of the tumor has been successful, and at the present time the growth has been carried through 6 generations by serial transfer.

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EXPLANATION OF PLATES

Plate 28

Fig. 1. Section of the uterus showing a small pedunculated mass of atypical glandular tissue. Hematoxylin and eosin. × 11.

Fig. 2. Section of the uterus showing a small nodule of actively growing tumor confined to the endometrium. Hematoxylin and eosin. × 11.

Fig. 3. Uterus opened opposite mesometrial insertion. Both horns contain tumor masses. The left horn is widely involved. The nodules arise from multiple points and no normal endometrium is visible. The right horn contains one large mass and a number of smaller nodules. × 1.1. Arrows indicate left horn (L), right horn (R), upper portion of vaginal canal (V) and various tumor nodules (T).

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Photographed by J. A. Carlile

(Greene and Saxton: Uterine adenomata in the rabbit. I)

PLATE 29

FIG. 4. Section of endometrium showing a group of atypical alveoli. Hematoxylin and eosin. × 133.

FIG. 5. Section of the pedunculated mass pictured in Fig. 1. Irregularly sized alveoli lined by atypical epithelium are scattered throughout an abundant myxoid stroma. Hematoxylin and eosin. × 165.

FIG. 6. Section of tumor nodule shown in Fig. 2. Stroma is scanty and coiled branching processes, papillary proliferations and interlacing bands tend to obscure the alveolar structure of the growth. Hematoxylin and eosin. × 165.

FIG. 7. Section of uterine wall showing invasion of the muscular coat. Hematoxylin and eosin. × 110.

Photographed by J. A. Carlile

(Greene and Saxton: Uterine adenomata in the rabbit. I)

PLATE 30

FIG. 8. Section of early uterine nodule obtained when the mass first became clinically palpable. The growth has invaded the wall of a blood vessel and detached tumor cells lie free in the lumen. Hematoxylin and eosin. × 275.

FIG. 9. Section of lung showing large metastatic nodule. Hematoxylin and eosin. × 140.

FIG. 10. Section of thyroid gland showing closely packed acini lined by low epithelium and an adjacent area containing numerous dilated intercommunicating glands and a portion of a macroscopic cyst. Hematoxylin and eosin. × 110.

FIG. 11. Section of suprarenal. A zone of more or less homogeneous material entirely separates cortex and medulla. Hematoxylin and eosin. × 49.

FIG. 12. Eye showing actively growing transplant in the anterior chamber. × $\frac{3}{4}$.

Photographed by J. A. Carlile

(Greene and Saxton: Uterine adenomata in the rabbit. I)

Plate 31

Fig. 13. Section of transplanted tumor in the eye resulting from the 2nd serial transfer. The tissue was obtained by biopsy 2 months after transplantation. Hematoxylin and eosin. × 67.

Fig. 14. Section of transplanted tumor in the eye resulting from the 6th serial transfer. The tissue was obtained by biopsy 1½ months after transplantation and shows a more compact cellular structure. Hematoxylin and eosin. × 67.

Fig. 15. Section of transplanted tumor in the testicle resulting from the 2nd serial intratesticular transfer. The original intratesticular transplant was derived from the 5th serial eye transfer. The tissue shown in this section was obtained by biopsy 1¾ months after transplantation. The growth is compact and very cellular. At one point perivascular spaces have been invaded. Hematoxylin and eosin. × 68.

Fig. 16. Section of a tumor nodule in a different portion of the same testicle. Hematoxylin and eosin. × 52.

Fig. 17. High power view of advancing edge of nodule pictured in Fig. 16. The section shows invasion of the testicular parenchyma with groups and columns of tumor cells surrounding and destroying tubules. Hematoxylin and eosin. × 267.

Photographed by J. A. Carlile

(Greene and Saxton: Uterine adenomata in the rabbit. I

ON CROSS REACTIONS OF IMMUNE SERA TO AZOPROTEINS

II. Antigens with Azocomponents Containing Two Determinant Groups

By K. LANDSTEINER, M.D., and J. van der SCHEER

(From the Laboratories of The Rockefeller Institute for Medical Research)

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The question of serological cross reactions has recently been commented upon by other authors and ourselves (1–5). To recapitulate briefly, an immune serum may exhibit cross reactions by virtue of an antibody able to combine with substances more or less closely related to the homologous antigen in chemical structure, or it may contain multiple antibodies, differing in specificity, some of which cross react with certain heterologous antigens. The appearance of several antibodies after immunization with a particular antigenic material may depend upon the presence in the latter of different antigenic molecules, or upon the existence, in a single molecule, of more than one determinant group; moreover, as has been shown in our studies on azoproteins (4), multiple antibodies varying somewhat in specificity may be produced in response to one determinant structure in cases where the antigen does not contain divers chemical groupings that in part are shared by the reacting heterologous antigens. To illustrate the latter, an immune serum produced by injecting an azoprotein made from *m*-aminobenzenesulfonic acid was found to contain several antibody fractions reacting differently towards antigens made from *o*-aminobenzenesulfonic, *m*-aminobenzoic and *m*-aminophenylarsenic acids. Similar results were found in other instances and may well be expected to apply to natural antigens and their antibodies.

One can assume therefore that, by and large, in using any immune serum one deals not with a single antibody but with a mixture of somewhat different immune bodies (*cf.* Heidelberger and Kendall (6), Goodner and Horsfall (7 *a*), Lee *et al.* (7 *b*), Hooker (2)). In the case of

multiple antibodies produced by single natural antigens, the familiar idea is that these antibodies are formed in response to and are indicative of different determinant groups in the antigenic molecule.

Evidence for this concept is offered by the observation that immune sera engendered by azoproteins contain different antibodies severally acting on the original protein, on the hapten and, probably, on the conjugate[1] (1, 8, 4).

Another example, somewhat better defined with regard to the underlying determinant structure in the antigen, is the following in which the antigen used was ovalbumin to which had been attached two sorts of determinant groups. Crystalline egg albumin was treated with picryl chloride (9) in order to introduce picryl radicals, and the product of the reaction was coupled in the usual manner with a diazonium compound, namely diazotized *p*-aminophenylarsenic acid. Following injection of this material into rabbits two sorts of antibodies were demonstrable by absorption experiments, one reacting with picryl proteins, the other with azoproteins made from *p*-aminophenylarsenic acid. Similar results would probably be obtained if two or more azocomponents were to be attached to protein. Antibodies combining with both the phenylarsenic acid and the trinitrophenyl (picryl) residues could not be definitely ascertained.

Since in these instances the structure of the antigens with regard to the relation of the determinant groups to each other is not fully established, there still remained for investigation the antibody response to simple haptens of known constitution possessing different groups each of which has been shown to serve as a serological determinant. With this in view, aromatic amino compounds containing two different acid groupings were synthesized and used for preparing conjugated antigens. The immune sera obtained by injection of these azoantigens were examined for the presence of multiple antibodies by means of "partial" antigens made from compounds containing only one of the acid groups.

Materials and Methods

3-Amino, 5-Carboxysuccinanilic Acid.—3,5-Dinitrobenzoic acid was reduced in alcoholic solution with ammonium sulfide by the method of Flürscheim (10) to 3-amino, 5-nitrobenzoic acid. For purification an aqueous solution of the ammonium salt was strongly acidified by adding concentrated HCl and after filtration the free amino compound was precipitated by addition of the required amount of NaOH. M.p. after recrystallization from water 208°C. (11).

[1] This might be taken to indicate the existence, in such sera, of different binding groups in a single antibody.

3-Amino, 5-nitrobenzoic acid and succinic acid were fused together for 15 minutes at 165° (12). The precipitate obtained by acidification of an alkaline aqueous extract of the product was recrystallized from water. Yield 70 per cent of theory. Needles, m.p. 212–213°C. Titration: 0.280 gm. neutralized 9.9 cc. N/10 NaOH. Calculated for 3-nitro, 5-carboxysuccinanilic acid ($C_{11}H_{10}O_7N_2$) 9.92 cc.

This compound was reduced with ferrous sulfate as described previously (12). After filtration the solution was made weakly acid to Congo red and the precipitate was recrystallized from water. Yield 75 per cent. Needles, m.p. 139–140°C. After drying at 50° *in vacuo* over $CaCl_2$: calculated for 3-amino, 5-carboxysuccinanilic acid ($C_{11}H_{12}O_5N_2$): N 11.11; found 11.06.

m-Aminosuccinanilic Acid.—*m*-Nitrosuccinanilic acid, prepared by fusion of *m*-nitraniline and succinic acid, was reduced with ferrous sulfate to the amino compound as described for the para compound (12). Yield 70 per cent of theory. Needles, m.p. 152°C. after recrystallization from water. Calculated for $C_{10}H_{12}O_3N_2$: N 13.45; found 13.37.

(3-Amino, 5-Succinylaminobenzoyl)-p-Aminobenzoic Acid.—Following the methods described below for the preparation of the corresponding As compounds (3,5-dinitrobenzoyl)-*p*-aminobenzoic acid was obtained by treating *p*-aminobenzoic acid with 3,5-dinitrobenzoyl chloride. After recrystallization from 50 per cent acetone (needles, no m.p. up to 250°C.), it was reduced by means of ammonium sulfide to (3-nitro, 5-aminobenzoyl)-*p*-aminobenzoic acid, which was recrystallized by acidifying a hot solution of the sodium salt in 50 per cent alcohol with a slight excess of N HCl and cooling to room temperature. Orange colored platelets, no m.p. up to 250°C. Calculated for $C_{14}H_{11}O_5N_3$: N 13.90; found 13.71.

A finely ground mixture of 3 gm. of this substance and 6 gm. of succinic anhydride, was kept at 130–135°C. for 15 minutes. The reaction mixture was dissolved in hot water by addition of sodium hydroxide and the substance precipitated by acidification with hydrochloric acid. It was recrystallized from a large volume of 50 per cent alcohol. Yield 2.6 gm. Clusters of needles, no m.p. up to 250°C. Calculated for (3-nitro, 5-succinylaminobenzoyl)-*p*-aminobenzoic acid ($C_{18}H_{15}O_8N_3$): N 10.47; found 10.51.

This nitro compound was reduced with ferrous sulfate and precipitated from the filtered solution by making weakly acid to Congo red. Recrystallized from a large volume of 50 per cent alcohol. Yield 75 per cent. Needles, decomposes at about 250°C. Calculated for $C_{18}H_{17}O_6N_3$: N 11.32; found 11.23.

(3-Amino, 5-Succinylaminobenzoyl)-p-Aminophenylarsenic Acid.—To 11 gm. of arsanilic acid dissolved in 300 cc. of 10 per cent sodium bicarbonate solution 17 gm. of 3,5-dinitrobenzoyl chloride dissolved in 200 cc. of chloroform were added in 5 portions with vigorous shaking during 1 hour. The precipitate formed upon acidification with HCl was dried and extracted several times with ether. Yield 19 gm. It was purified further by dissolving in 1 liter of boiling water and 20 cc. of concentrated ammonium hydroxide, and precipitating with acetic acid. Needles. Calculated for (3,5-dinitrobenzoyl)-*p*-aminophenylarsenic acid ($C_{13}H_{10}O_8N_3As$): N 10.22; found 10.12.

The substance was dissolved in hot water by addition of a slight excess of ammonia, and there was added 90 per cent of the quantity of ammonium sulfide (see Flürscheim (10)) required for the reduction of one nitro group. After being heated for 1 hour the solution was evaporated to dryness *in vacuo* and the residue was extracted with boiling water. To the extract was added one-fifth volume of concentrated HCl, and after cooling in a freezing mixture for ½ hour it was filtered and adjusted to weak acidity to Congo red by addition of concentrated NaOH. The precipitate was recrystallized from a large volume of 30 per cent alcohol. Yield 6 gm. from 10 gm. of the dinitro compound. Clusters of needles. Calculated for (3-nitro, 5-aminobenzoyl)-*p*-aminophenylarsenic acid ($C_{13}H_{12}O_6N_3As$): N 11.02; found 10.94.

10 gm. of the nitroamino compound were finely ground and refluxed for 3 hours with 150 cc. of absolute alcohol and 10 gm. of succinic anhydride. After cooling the material was filtered off and freed from unchanged nitroamino compound by dissolving in 200 cc. water by means of NaOH and subsequently adding 60 cc. of concentrated HCl, the amino compound staying in solution. The precipitate was purified after it had been dissolved in a small amount of water with NaOH by precipitation with HCl and crystallization from a large volume of 50 per cent alcohol. Yield 6 gm. Oval shaped platelets. Calculated for (3-nitro, 5-succinylaminobenzoyl)-*p*-aminophenylarsenic acid ($C_{17}H_{16}O_9N_3As$): N 8.73, As 15.57; found N 8.74, As 15.34.

4 gm. of the above substance were finely ground and suspended in a mixture of 100 cc. of 95 per cent ethyl alcohol and 100 cc. of N HCl, and 8 gm. of zinc dust were slowly added with continuous stirring over a period of about 1 hour with further addition of 20 cc. of N HCl when the solution became weakly acid. After the nitro compound had disappeared the solution was filtered and 8 gm. of sodium acetate were added. The precipitate was washed twice with water and redissolved in 100 cc. of water by addition of N NaOH. The solution was made strongly acid to Congo red by addition of concentrated HCl and clarified by centrifugation. After neutralization of the solution and addition of sufficient ammonia to redissolve the precipitate formed, ammonium sulfide was added until a flocculent precipitate of zinc sulfide was formed. After removal of zinc sulfide acidification to weak Congo red reaction gave an amorphous precipitate which crystallized upon heating on the steam bath (rosettes of oval platelets). Yield 2.4 gm. For further purification 1 gm. of substance was dissolved in 50 cc. of water by addition of N NaOH; the neutral solution was decolorized by heating with charcoal. The hot filtrate was acidified with 5 cc. glacial acetic acid; crystallization took place slowly upon cooling. Calculated for (3-amino, 5-succinylaminobenzoyl)-*p*-aminophenylarsenic acid ($C_{17}H_{18}O_7N_3As$): N 9.31, As 16.60; found N 9.15, As 16.48.

sym. Aminoisophthalyl Glycine-d,l-Leucine.—A solution of 5 gm. of sym. nitroisophthalic acid in 100 cc. of dry ether was cooled to 0° and shaken with 5 gm. of finely ground PCl_5 for about 45 minutes at low temperature until all the PCl_5 was used up. After evaporation to dryness at 60° under reduced pressure the

residue was extracted twice with 25 cc. of boiling benzene. Addition of 3 volumes of dry petrol ether to the benzene solution gave a crystalline precipitate of the monochloride which was recrystallized from 20 parts of dry toluene. Yield 1.2 gm. Platelets, m.p. 123–124°, with evolution of gas. Calculated for sym. nitroisophthalic acid monochloride ($C_8H_4O_5NCl$): Cl 15.44; found 15.29.

To a dry chloroform solution (50 cc.) of glycine ethyl ester prepared from 5 gm. of hydrochloride were added 5 gm. of dry dimethylaniline and 5 gm. of sym. nitroisophthalic acid monochloride dissolved in 125 cc. of dry chloroform. The mixture was allowed to stand overnight at room temperature and after removing a precipitate of glycine ester hydrochloride the solution was shaken with 200 cc. of N/2 HCl. The precipitate was filtered off, washed with water and recrystallized from 50 per cent alcohol. Yield 5 gm. Clusters of needles, m.p. 203–204°C. Calculated for sym. nitroisophthalyl monoglycine ethyl ester ($C_{12}H_{12}O_7N_2$): N 9.46; found 9.37.

2.95 gm. of sym. nitroisophthalyl monoglycine ethyl ester in a mixture of 60 cc. of dry benzene and 20 cc. of dry ether were shaken with 3 gm. of PCl_5 for ½ hour at room temperature and finally a short time at 37°C. When practically all the PCl_5 had disappeared the solution was filtered and the substance was precipitated by adding 3 volumes of dry petroleum ether. Yield 2.4 gm. Platelets, m.p. 127–128°C. Calculated for chloride of sym. nitroisophthalyl monoglycine ethyl ester ($C_{12}H_{11}O_6N_2Cl$): Cl 11.29; found 11.36.

A solution of 3 gm. of this substance in 50 cc. of dry ethyl acetate was mixed with a solution of *d,l*-leucine methyl ester, prepared from 3.5 gm. of hydrochloride, in 75 cc. of dry ethyl acetate. After standing at room temperature overnight the solution was shaken successively with dilute HCl, dilute aqueous $NaHCO_3$ and water. After drying with Na_2SO_4 the ethyl acetate was removed by evaporation and the syrup was dissolved in 40 cc. of methyl alcohol. 20 cc. of N NaOH were added and the saponification was allowed to proceed for 1 hour at room temperature. After neutralization the solution was evaporated to dryness, the residue was dissolved in a small volume of water and made acid to Congo red by addition of 5 N HCl. After several days in ice box and occasional rubbing, the sticky precipitate crystallized in hair-like needles. The substance was redissolved in water with alkali and reprecipitated with acid. It was filtered off in the ice box and was dried *in vacuo* over P_2O_5. Yield 3 gm. M.p. 125–126°C. Calculated for sym. nitroisophthalyl glycine-*d,l*-leucine ($C_{16}H_{19}O_8N_3$): C 50.39, H 5.02, N 11.02; found C 50.48, H 5.08, N 10.90.

3.8 gm. of the nitro compound dissolved in 10 cc. of water by addition of ammonia were added to a hot solution of 18.7 gm. of ferrous sulfate (7 aq.) in 50 cc. of water. 16 cc. of 28 per cent ammonia solution were added in 5 portions over a period of 10 minutes. After ½ hour's heating on the steam bath, the solution was filtered and evaporated *in vacuo* to about 25 cc.; a large volume of alcohol was added. Ammonium sulfate was removed by filtration and the solution was evaporated *in vacuo*. After redissolving the residue in absolute methyl alcohol and evaporating to dryness, the substance was taken up in 30 cc. of absolute

methyl alcohol. The solution was filtered and the substance precipitated with 90 cc. of dry ether. Upon dissolving the precipitate in a large amount of boiling absolute ethyl alcohol and adding sufficient ether to the cooled solution to give a slight turbidity, crystallization took place in the ice box. The free amino compound obtained by acidification of a concentrated aqueous solution of the ammonium salt was a syrup which did not crystallize. It was washed several times with cold water and solidified on drying *in vacuo* over P_2O_5. Calculated for sym. aminoisophthalyl glycine-*d,l*-leucine ($C_{16}H_{21}O_6N_3$): C 54.66, H 6.07, N 11.95; found C 54.50, H 6.19, N 11.67. Titration: 0.0667 gm. in 50 per cent alcohol neutralized 18.85 cc. N/50 $Ba(OH)_2$. Calculated 19.00 cc.

sym. Aminoisophthalyl Glycine-d,l-Phenylalanine.—The chloride of sym. nitroisophthalyl glycine ethyl ester was condensed with the ethyl ester of *d,l*-phenylalanine in dry ethyl acetate solution as described above. After washing the solution with dilute HCl, etc., and drying over Na_2SO_4, it was evaporated to dryness *in vacuo* and the residue was recrystallized from methyl alcohol. Yield 3.8 gm. from 3 gm. of the chloride. Hair-like needles, m.p. 138°C.

To a solution of 3.7 gm. of this ester in 70 cc. of acetone were added 16 cc. of N NaOH, and after 1 hour the solution was neutralized and evaporated to dryness. The residue was dissolved in a small amount of water and after acidification to Congo red the oil which separated was stirred with chloroform until crystallization took place. After acidification of a very dilute alkaline aqueous solution and removal of a small amount of tarry material the substance crystallized slowly at room temperature. Yield 2.5 gm. Clusters of curved needles, m.p. 211–212°C. Calculated for sym. nitroisophthalyl glycine-*d,l*-phenylalanine ($C_{19}H_{17}O_8N_3$): N 10.11; found 10.01.

The reduction of the nitro compound was carried out with ferrous sulfate as described above. After removing most of the ammonium sulfate with alcohol, the solution was evaporated to dryness and the residue dissolved in a small amount of water. Upon acidification an oil separated which slowly crystallized in microscopic platelets. The substance was recrystallized from a large volume of water. After drying at 90° *in vacuo* it softened at 139°, and decomposed at 178°. Calculated for sym. aminoisophthalyl glycine-*d,l*-phenylalanine ($C_{19}H_{19}O_6N_3$): N 10.90; found 11.03.

m-Nitrobenzoyl and m-Aminobenzoyl Derivatives of Glycine and d,l-Leucine.—These were prepared by shaking a solution of the amino acids in 10 per cent $NaHCO_3$ (the leucine was first dissolved in water by addition of a slight excess of N NaOH) with a chloroform solution of *m*-nitrobenzoyl chloride (1.25 mols to each mol of amino acid) and reduction of the nitro compounds with ferrous sulfate as described previously.

m-Nitrobenzoyl glycine: Recrystallized from 50 per cent alcohol. Needles, m.p. 165–166°C. Titration: 200 mg. dissolved in 50 per cent alcohol required for neutralization 8.9 cc. N/10 NaOH. Formula $C_9H_8O_5N_2$ requires 8.92 cc.

m-Aminobenzoyl glycine: Recrystallized from water. Platelets, m.p. 191–192°C. Calculated for $C_9H_{10}O_3N_2$: N 14.43; found 14.25.

m-Nitrobenzoyl *d,l*-leucine: Recrystallized from benzene. Long needles, m.p. 132–133°. Titration: 200 mg. dissolved in 50 per cent alcohol required for neutralization 7 cc. N/10 NaOH. Formula $C_{13}H_{16}O_5N_2$ requires 7.14 cc.

m-Aminobenzoyl *d,l*-leucine: Recrystallized from water. Platelets, m.p. 157–158°C. Calculated for $C_{13}H_{18}O_3N_2$: N 11.19; found 11.12.

Immunization.—Rabbits were injected intravenously with 2 cc. of a suspension containing 2.5 mg. of azostromata (from horse blood) (4) in 1 cc. After two to four courses of 6 daily injections given at intervals of 1 week, the animals were bled 7 days following the final injection.

Tests.—Antigens used for the tests were made by coupling the diazonium compounds with chicken serum as described (13). The dilutions of the test antigens given in the tables are in terms of a 5 per cent stock solution. The intensity of the reactions is indicated as follows: 0, f.tr. (faint trace), tr. (trace), tr. (strong trace), ±, ∓, +, +±, ++, etc.

Absorption Experiments.—A suspension of azostromata was centrifuged and the sediment was mixed with concentrated or diluted immune serum. The suspension was stirred occasionally during 2 hours at room temperature, after which the stromata were removed by centrifuging (4).

EXPERIMENTAL[2]

The sera made with two of the four antigens, those from aminocarboxysuccinanilic acid (SC) and (aminosuccinylaminobenzoyl)-*p*-aminobenzoic acid (SB), contained, curiously, antibodies that were entirely or almost completely directed towards only one of the acid groups, namely the succinanilic acid residue, and therefore did not yield the desired information on the alternative whether there would be formed antibodies corresponding to the structure of the molecule as a whole or more than one antibody, each adjusted to one of the determinant groups. Thus the SC antisera precipitated with nearly equal intensity the SC azoprotein and an azoprotein made from *m*-aminosuccinanilic acid (S) but out of four SC sera only one, not the most potent, gave a weak reaction with an azoprotein prepared from *m*-amino-

[2] For brevity the substances used for preparing the azoantigens, as well as these antigens and the corresponding antisera, will be designated throughout as follows: 3-amino, 5-carboxysuccinanilic acid (SC), *m*-aminosuccinanilic acid (S), (3-amino, 5-succinylaminobenzoyl)-*p*-aminobenzoic acid (SB), (3-amino, 5-succinylaminobenzoyl)-*p*-aminophenylarsenic acid (SA), *p*-aminophenylarsenic acid (A), sym. aminoisophthalyl glycine-*d,l*-leucine (GIL), sym. aminoisophthalyl glycine-*d,l*-phenylalanine (GIPh), *m*-aminobenzoyl glycine (G), *m*-aminobenzoyl *d,l*-leucine (L).

benzoic acid. The relation in chemical structure of the compounds is seen from the formulae given below.

Although directed towards the succinanilic acid group of the SC antigen, the SC immune sera were found by inhibition tests not to be identical with sera made with S antigen. In such tests the reactions of SC sera on SC or S antigens were markedly more inhibited by 3-nitro, 5-carboxysuccinanilic acid than by *m*-nitrosuccinanilic acid, while this was not the case with S immune sera. The homologous reaction of SC sera was not significantly inhibited by *m*-nitrobenzoic acid.

TABLE I

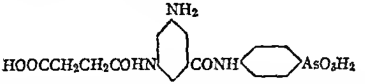
0.1 cc. of the immune sera was added to 0.2 cc. of the antigen solutions. Readings after 1 hour at room temperature.

Immune sera	Test antigens														
	SA			S			A			SC			SB		
	1:100	1:500	1:2500	1:100	1:500	1:2500	1:100	1:500	1:2500	1:100	1:500	1:2500	1:100	1:500	1:2500
SA	+±	+++	+	+++	++++	+	tr.	++	+	++±	+++	+	++	+++	+
S	f.tr.	±	f.tr.	+±	+++	±	0	0	0	+	+±	±	tr.	±	f.tr.
A	0	±	tr.	0	0	0	±	++±	+	0	0	0	0	0	0
SC	f.tr.	+	±	+	+++	+	0	0	0	+	++±	+±	tr.	+	tr.

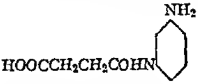
Essentially similar in all respects were the results observed with (3-amino, 5-succinylaminobenzoyl)-*p*-aminobenzoic acid antigen. The sera failed to precipitate *p*-aminobenzoic acid antigen but reacted with S antigen, even more intensely than with SB antigen, while immune sera made with *p*-aminobenzoic acid antigen gave distinct precipitation with SB antigen. Inhibition tests as described above again demonstrated the greater affinity of the homologous substance and clearly showed a difference between SB and S immune sera.

In distinction to the experiments described, results answering the question under discussion were obtained with two other antigens, namely SA and GIL azoproteins. Immune sera for SA gave precipitation with the homologous antigen and also with antigens containing only one of the residues, either succinanilic or phenylarsenic acid. S and A as well as SC sera reacted considerably better with the corre-

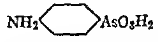
sponding than with SA (or SB) antigen. The tests are presented in Table I. The structure of the compounds used in these and the above experiments is shown in the formulae:



(3-amino, 5-succinylaminobenzoyl)-*p*-aminophenylarsenic acid (SA)



m-aminosuccinanilic acid (S)

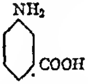


p-aminophenylarsenic acid (A)

(3-amino, 5-succinylaminobenzoyl)-*p*-aminobenzoic acid (SB)

p-aminobenzoic acid

3-amino, 5-carboxysuccinanilic acid (SC)



m-aminobenzoic acid

The presence of two distinct and unrelated sorts of antibodies could be shown by absorption with antigens (azostromata) that contained but one of the two determinant groups. Each of the "partial" antigens was seen to remove antibodies acting on its own determinant structure without affecting those for the other group. This held true even on repeated absorptions (Tables II*a* and II*b*).

Like absorption experiments, giving confirmatory results, were carried out on a second SA serum using A, SB and S antigens. After absorption with both antigens, A and S (or A and SB), the immune sera no longer gave any reaction with the homologous antigen SA.

Immune sera produced to an antigen containing the two amino acids, glycine and leucine, linked to one benzene ring also showed the presence of two distinct sorts of antibodies each directed towards one of the two determinants. Precipitin tests showing the cross reactions

TABLES II*a* AND *b*

SA immune serum was absorbed separately with SB and with A azostromata for 2 hours at room temperature, 8 mg. of azostromata being used for 3 cc. of undiluted immune serum. In each case a portion of the absorbed immune serum was treated similarly with a proportionate amount of the same azostromata; a third absorption was carried out in the same manner. For the tests 0.2 cc. of the absorbed fluids (designated in the tables as I, II and III) was mixed with 0.05 cc. of the test antigens (dilution 1:100 of a 5 per cent solution). Readings were taken after 1 hour at room temperature (first line) and after standing overnight in the ice box (second line).

TABLE II*a*

Repeated Absorptions with SB

	Test antigens		
	SA	SB	A
I	+± +++	0 0	++± ++++
II	+± +++	0 0	++± ++++
III	+± ++±	0 0	++± ++++
Unabsorbed immune serum	+++ ++++	+++ ++++	++± ++++

TABLE II*b*

Repeated Absorptions with A

	Test antigens		
	SA	SB	A
I	+++ ++++	+++ ++++	0 0
II	+++ ++++	+++ ++++	0 0
III	+++ +++±	+++ +++±	0 0
Unabsorbed immune serum	+++ ++++	+++ ++++	++± ++++

TABLE III

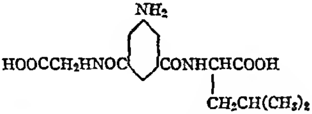
0.1 cc. of the immune sera was added to 0.2 cc. of the antigen solutions. Readings after 1 hour at room temperature.

Immune sera	Test antigens											
	GIL			G			L			GIPh		
	1:100	1:500	1:2500	1:100	1:500	1:2500	1:100	1:500	1:2500	1:100	1:500	1:2500
GIL	+±	++±	+±	±	+	±	±	+±	±	+±	+±	tr.
G	0	±	±	+±	++±	+	0	0	0	tr.	±	±
L	±	+	±	0	f.tr.	f.tr.	tr.	++±	+			
G + L*	f.tr.	+	±	±	++	+	0	+±	±			
G + L†	tr.	+	±	±	+±	+	tr.	++	+			

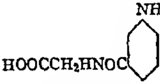
* Mixture of equal parts of G and L immune serum.

† Mixture of one part of G immune serum and two parts of L immune serum.

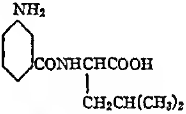
of GIL, G and L immune sera on the antigens with one or both determinants are presented in Table III, where in addition a comparison is made of GIL serum with mixtures of G and L immune sera. One example only is given for each kind of sera; the results with additional immune sera were in agreement.



sym. aminoisophthalyl glycine-leucine (GIL)



m-aminobenzoyl glycine (G)



m-aminobenzoyl leucine (L)

sym. aminoisophthalyl glycine-phenylalanine (GIPh)

The separation of two sorts of antibodies in GIL serum by absorption is demonstrated in Tables IV*a* and *b*, there being stronger reactions on the leucine than on the glycine moiety. When the immune serum was treated with a mixture of G and L azostromata, the reaction with the "full" antigen became weak after two absorptions but there was still a faint reaction after the third absorption which may perhaps indicate the presence of a slight amount of a special antibody (Table V).

From the tests with mixed G and L immune sera (Table III) the inference may be drawn that the G and L antibodies found in the GIL serum are not the same as the antibodies present in G and L sera, for it

TABLES IV *a* AND *b*

GIL immune serum was absorbed separately with G and with L azostromata for 2 hours at room temperature, using 4.5 mg. of azostromata and 3 cc. of diluted immune serum (1:2). This procedure was repeated twice and tests were made with the fluids as in Tables II *a* and *b*.

TABLE IV *a*

Repeated Absorptions with G

	Test antigens		
	GIL	G	L
I	++± +++±	0 0	++ ++±
II	++ +++±	0 0	+± ++±
III	++ +++±	0 0	+± ++±
Unabsorbed immune serum	+++ +++±	+ +	++ ++±

TABLE IV *b*

Repeated Absorptions with L

	Test antigens		
	GIL	G	L
I	++ ++±	+ +	0 0
II	+± ++	+ +	0 0
III	+ ++	+ +	0 0
Unabsorbed immune serum	+++ +++±	+ +	0 0

TABLE V

Repeated Absorption with G + L

GIL immune serum was absorbed with a mixture of G and L azostromata for 2 hours at room temperature, using 3 mg. azostromata of each kind for 3 cc. of immune serum (diluted 1:2 with saline). This procedure was repeated twice and the tests were made with the fluids (I, II and III) as above.

	Test antigens		
	GIL	G	L
I	+ +±	0 0	0 tr.
II	tr. ±	0 0	0 0
III	f.tr. tr.	0 0	0 0
Unabsorbed immune serum	+++ +++±	+ +	++ +++±

appears that a mixture of the latter gives weaker precipitation with GIL antigen than do GIL immune sera whereas this is not the case in tests with G or L antigens. Inhibition experiments supported the above conclusion in so far as the antibodies in GIL serum reacting on G or L antigens were slightly better inhibited by GIL than by G or L; on the other hand, with G and L immune sera there was a difference in the opposite direction.

COMMENT

The experiments made with sera for the compounds containing both glycine and leucine (GIL), or succinic and phenylarsenic acid (SA) residues definitely establish the existence of cases in which discrete antibodies are formed that are individually directed towards separate determinant groups in one substance, even though it be of small molecular size and the determinant groups rather closely adjoining. The failure of repeated absorption with an antigen which contains only one of the two groupings, to effect a significant diminution of the antibody reacting with the other group, is proof for the lack of serological kinship between the two antibodies demonstrated. It is in contrast to the behavior of multiple but related antibodies developed through the stimulus of a single determinant structure, where continued absorption with a reacting heterologous antigen was often seen to exhaust the immune serum completely (4).[3] It may be noted that, in the substances here examined, the determinant groups are acid, namely the residues of succinic acid, phenylarsenic acid, glycine and leucine which have been shown to exert a prominent influence on specificity. Furthermore, comparative experiments with antisera produced by antigens with one acid group showed that the presence of a second group produced a qualitative change in the antibodies (*cf.* Morgan (3), (5)).

There were practically no antibodies in the sera examined which were not removable by absorption with both "partial" antigens. Also, antibodies that have two combining groups corresponding respectively to the two determinants of the substances investigated could not be demonstrated, and appear to be present in the GIL and SA immune sera, if at all, only in small quantities since, as just mentioned, exhaus-

[3] No attempts have been made in the present investigation to separate such fractions by absorption with suitable antigens.

tion with one partial antigen did not diminish the reaction for antigens containing the other determinant. Yet evidence, although calling for more extensive investigation, of the existence of such combining groups of different specificities in an antibody molecule[4] may perhaps be derived, as mentioned, from some observations on antibodies which appear to react at the same time with the protein and the hapten parts of azoproteins.

The results discussed, particularly the formation of antibodies against parts of the haptens studied, are connected with the issues debated lately of the univalence or multivalence of antibodies and the size of their combining areas. A relatively small specific site is indicated by the attachment of several antibody molecules to one molecule of antigen (*cf.* Haurowitz (8)), and may also explain why, remarkably, the various antibodies, both chemically and in their specificity, when employed as antigens, are rather similar to one another and, as well, to globulins present in normal sera. In this connection, however, it should be considered that the specificity of antibodies, or else their activity, may well depend on structures of a different character from those underlying antigen specificity; in fact, the specific properties of antibodies appear in general to be entirely destroyed by denaturation through heat and other agents, while denaturation causes alterations of comparatively lesser degree in the specific serological properties of protein antigens.

In the case of immune sera to peptides (16) or disaccharides (17), there has not been demonstrated so far the resolution of the whole immune body content into antibody fractions related to definite parts of the molecules such as these encountered in the present experiments. Future studies will be necessary to determine the structural features requisite for the production of antibodies severally directed towards different groups in one molecule and to ascertain to what extent such antibodies occur in immune sera prepared by means of natural antigens and how far they are responsible for cross reactions (*cf.* 4).[5] Of some significance, too, for the problem of immune bodies to natural antigens are the two instances reported, in which an appreciable amount of anti-

[4] See also Morgan (14), and Meyer (15).

[5] Since this paper was completed there has appeared a study by Marrack and Carpenter (18) in which this question is discussed with reference to the cross reactions of polysaccharides.

bodies was produced for one, only, of the two groups present though each had formerly been found to serve as a serological determinant.

The experiments were carried out with the assistance of Mr. B. Meier.

SUMMARY

Azoproteins have been prepared with azocomponents possessing two serologically active groups. On immunization with such antigens immune sera were obtained containing two separate, unrelated antibodies, each specific for one of the two groups and separable by absorption. In other cases one of the two structures was dominant, in that antibodies were formed only towards this and not towards the other grouping. The specificity of the antibodies was in general found to be influenced to some extent by the presence of a second group in the antigen. The relevancy of these observations for antibodies directed against natural antigens has been noted.

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STATISTICAL STUDIES OF THE NATURE OF THE INFECTIOUS UNIT OF VACCINE VIRUS

By ROBERT F. PARKER, M.D.

(From the Department of Medicine, Western Reserve University, School of Medicine, Cleveland)

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An infectious unit of vaccine virus is the smallest amount of the active agent which is capable of initiating infection when inoculated under certain conditions into a specified host. This definition does not depend upon any hypothesis as to the nature of the pathogenic agent, and likewise no assumption is made as to the physical state in which the virus exists. Thus the virus preparation might be a solution of an active protein, and Stanley (1) has shown that this is apparently true for tobacco mosaic virus. On the other hand, the virus might exist in particulate form with the particles distributed at random in the suspension. The inoculation of 1 or of a certain number of these particles might be required to cause infection. Several investigators have presented evidence that vaccine virus is in fact particulate.

Duran-Reynals (2) has observed that the inoculation of very dilute suspensions of vaccine virus intradermally in rabbits may give rise to a group of papules rather than to a single lesion. This occurs more frequently if the suspension contains a "spreading factor" which he has described. This he regards as evidence that the critical concentration of virus required for infection is a "single infectious unit," presumably an individual particle. Doerr (3) points out that when a virus suspension is titrated by inoculating serial dilutions of the stock suspension, it sometimes happens that a lesion follows the inoculation of a given dilution of virus but not of the next lower one which should theoretically contain 10 times as much virus. He shows that this paradox can most easily be explained if one assumes that the virus is particulate, and that a single particle can cause infection. He does not, however, develop the idea further. Burnet (4) recently has advocated the use of the chorio-allantoic membrane of chick embryos for titrating certain viruses, and Keogh (5) has used the technique in quantitative studies of vaccine virus. Suitably inoculated, this virus causes the development of discrete lesions in the chick membrane which may be counted. If a sufficient number of mem-

branes are inoculated with each dilution, and the average number of lesions determined, an arithmetic ratio may be shown to exist between the concentration of virus and number of lesions produced. Thus twofold dilution of the virus suspension leads to the production on inoculation of half as many lesions as were produced by inoculation of the original suspension. These workers consider, therefore, that each lesion arises as the result of the infection of a cell with a single particle of virus.

These observations constitute presumptive evidence that the virus of vaccinia is particulate and that a single particle of virus can give rise to infection. Evidence is presented by other workers that vaccine virus in a suspension is associated with elementary bodies, minute structures which under the proper conditions can be made visible (6). It becomes important, therefore, to determine whether a single elementary body can give rise to vaccinal infection, or whether in order to accomplish this several corpuscles must be introduced simultaneously.

Parker and Rivers (7) prepared suspensions of elementary bodies which were relatively free from other material. The number of infectious units per unit volume was determined by titration in rabbits, the number of particles by counting the particles in a small sample in a calibrated chamber. They showed that there was a high degree of correlation between the number of particles in a suspension and the number of infectious units. These data did not, however, permit conclusions as to the number of particles which composed an infectious unit of virus.

Since it was evidently impracticable to determine by direct observation the number of elementary bodies necessary to cause infection, a statistical method of approach was sought. The most promising of these seemed to be that elaborated by Greenwood and Yule (8) for application to water bacteriology and widely used since that time. The method was employed by Youden, Beale, and Guthrie (9) in studying the virus of tobacco mosaic, and these authors elaborated the formulae in order to take account of the number of sites available for infection.

The problem to be solved is simply a special case of the theory of probability. The principle involved was first clearly stated by Poisson (10) and is generally referred to as Poisson's law of small numbers. He showed that if successive samples were drawn from a universe which consisted of very small particles suspended in a liquid, the proportion of samples which contain 1, 2, 3 . . . *n* particles was related to the mean number of particles per unit volume in a definite manner.

He presented methods, moreover, by which that proportion could he calculated. It follows that if the mean number of particles per unit volume is known, and if an adequate number of samples is drawn, the proportion which will contain no particles, 1 or more, 2 or more, ...n or more particles can be calculated. It has heen shown by Greenwood and Yule that the proportion of samples containing no particles can be determined hy solving the simple equation $P = e^{-m}$, where P is the proportion of samples containing no particles, e is the hase for natural logarithms (2.718), and m is the mean number of particles. The proportion of samples

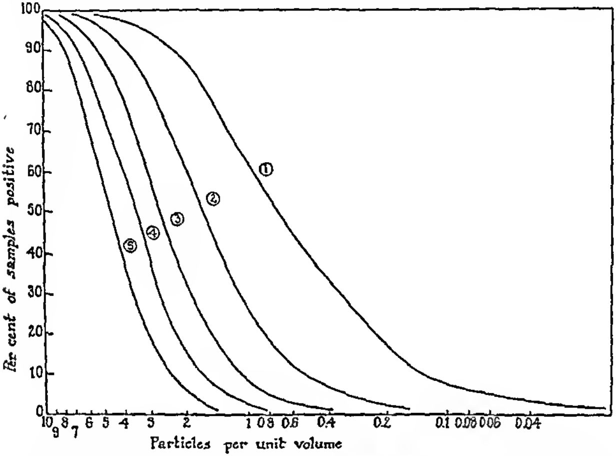

FIG. 1. Theoretical curves. Per cent of samples containing at least 1, at least 2.... particles plotted against mean concentration of particles. Figures on the curves indicate the minimum number of particles per sample required to classify the sample as positive.

containing 1 or more particles is therefore $1 - e^{-m}$. The calculation of the proportion of samples containing 2 or more particles is equally straightforward hut lahorious and the figures are more easily obtained from published tables of Poisson's exponential limit (11). If the probability of obtaining a sample containing 1 or more, 2 or more, 3 or more particles is plotted against various values of m, a characteristic curve is obtained in each case. Such curves are shown in Fig. 1, where it will be seen that as the number of particles required for success is increased the slope of the curve becomes progressively steeper. It is evident that if the conditions of experiment are such as to allow the result of an inoculation to be

interpreted as either positive or negative, experimental curves can be constructed and fitted to these theoretical ones. Other conditions then being suitable, it should be possible directly to determine the virulence of a virus even though one is not able to count the virus particles directly. The application of the principle to a broad range of biological problems has been suggested by Iwaszkiewicz and Neyman (12), who give derivations of their formulae.

EXPERIMENTAL

The theoretical considerations which have been outlined led us to believe that by the application of relatively simple statistical principles it should be possible to determine the number of particles—elementary bodies—of vaccine virus which are required to initiate infection in the rabbit skin. The present experiments were designed to test this hypothesis and to make use of the method to compare the characteristics of a number of strains of vaccine virus.

Materials and Methods

Virus.—Several strains of vaccine virus have been employed. They are the following:

1. Board of Health Strain.—This strain is that used by the New York City Board of Health and has been maintained for some time by testicular passages in rabbits. For the past year it has served in our laboratory for the routine production of elementary bodies of vaccinia. Inoculated intradermally in rabbits it causes typical lesions which have a moderate tendency to become hemorrhagic and necrotic. When many heavy inoculations have been given, generalized vaccinia usually results. Generalization does not commonly follow the multiple inoculation of small quantities of virus.

2. Cultured Vaccine Virus.—This strain, derived from the Board of Health strain, has been cultured in a chick embryo-Tyrode solution medium for many generations.[1] A complete description of its history and present characteristics will be found in the publications of Rivers and Ward (13). Suffice it to say that during prolonged cultivation its pathogenicity for the rabbit has altered markedly and it now causes relatively mild lesions in rabbit skin, which rarely become necrotic.

3. Noguchi Strain.—This strain was adapted to passage in the testicles of rabbits by Noguchi (14) and since has been maintained in the same manner. It causes generalization more often than the Board of Health strain.

Titration of Virus.—The virus was titrated by inoculating 0.25 cc. portions of successive serial dilutions of virus suspension intradermally into rabbits. The animals were observed daily and the presence of lesions recorded. Following

[1] In the laboratory of Dr. T. M. Rivers.

inoculation of Board of Health or of Noguchi virus, little trouble was experienced because of the appearance of "indeterminate" lesions. More difficulty was encountered with cultured virus because of the different character of the papules which followed inoculation of minimal amounts of virus. Many of these had a diameter of but 3 to 4 mm. and few showed evident necrosis. Nevertheless 9 out of 10 inoculations could usually be classified easily as positive or negative. In the remainder an element of judgment was of necessity admitted.

Source of Animals.—Rabbits were purchased in the open market and were of many breeds and mixtures. They weighed from 4 to 10 pounds; were selected on the basis only of being in a good state of nutrition and of having minimal areas of coarse skin. No animals which manifested detectable resistance to infection with vaccine virus were encountered.

The Board of Health strain of vaccine virus was chosen for the first experiment because it had been used in making preparations of elementary bodies as routine and because it causes well defined lesions in the rabbit skin.

Experiment 1.—The virus was used in the form of elementary bodies suspended in buffer solution. The suspension was centrifuged for 30 minutes at 2000 R.P.M. in order to ensure that the elementary bodies remaining in suspension would be well distributed, and in so far as possible without any clumps of particles being present. A rough titration was done by inoculating a rabbit with serial dilutions of suspension, 4 inoculations being made of each dilution. From this it was calculated that a virus dilution of $10^{-7.4}$ should give an equal number of positive and negative results. An exact titration was done as follows. A quantity of virus was diluted to $10^{-6.7}$ (5 times the concentration giving equal numbers of positive and negative results) in order to ensure that the lowest dilution inoculated would give rise to a high percentage of takes. From this suspension serial dilutions were prepared having a ratio of approximately 1–2 (log 0.3) and as soon as possible rabbits were inoculated therewith. The inoculations were completed within an hour and a half after the suspensions were prepared. Each animal was given an average of 45 inoculations, 5 or 6, therefore, of each dilution. Care was taken to distribute these so that the sites of inoculation of each dilution were distributed at various places over the rabbit. A single set of virus suspensions served for inoculation of all of the animals. The animals were observed daily.

The results of this experiment are presented graphically in Fig. 2. The calculations are collected in Table I. It will be observed that the experimental curve agrees very well with that calculated on the hypothesis that 1 particle is capable of causing infection since a deviation from the theoretical curve as great as the one observed would be expected to occur, owing to chance, in 9 of 10 trials. If

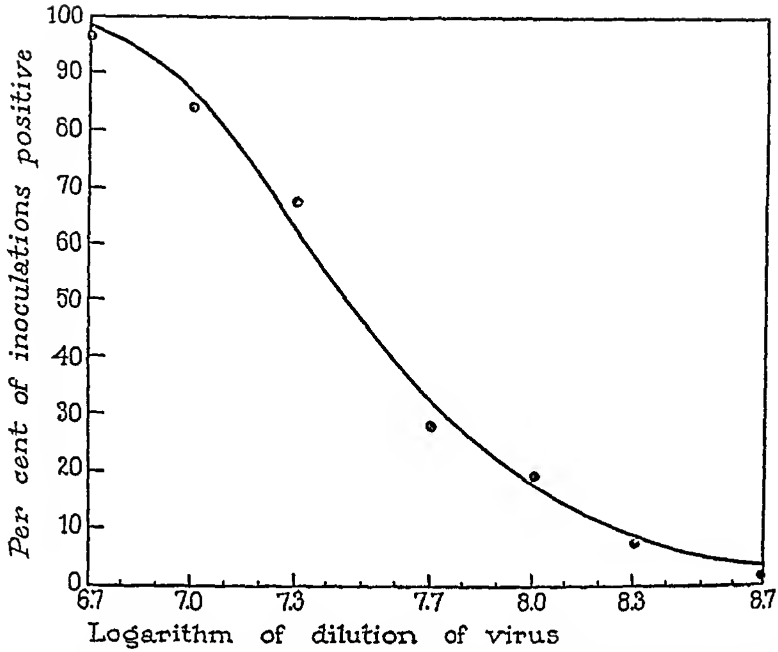

FIG. 2. Titration of Board of Health virus. Solid line represents theoretical curve, calculated on hypothesis that a single particle causes infection. Dots are experimentally determined points.

TABLE I

Experiment 1. Titration of Board of Health Virus. Calculations of Goodness of Fit of Experimental and Theoretical Curves

Logarithm of virus dilution	Results of experiment		If 1 particle initiates infection		If 2 particles initiate infection		If 3 particles initiate infection	
	Number of inoculations	Number positive (x)	Number expected positive ($\bar{x}$)	$\frac{(x-\bar{x})^2}{\bar{x}}$	Number expected positive	$\frac{(x-\bar{x})^2}{\bar{x}}$	Number expected positive	$\frac{(x-\bar{x})^2}{\bar{x}}$
6.7	69	67	67.5	0.004	68.3	0.025	69.0	0.06
7.0	69	58	59.3	0.028	57.7	0.002	67.5	1.36
7.3	72	49	44.6	0.433	48.0	0.208	50.2	0.03
7.7	71	20	22.8	0.345	16.1	0.947	13.6	3.02
8.0	72	14	13.0	0.076	6.5	8.650	2.5	49.80
8.3	75	6	6.7	0.073	1.3	15.00	0.6	48.70
8.7	76	1	3.0	1.330	0.5	0.500	0.2	3.20
			χ^2	2.28		23.33		106.2
			P	0.9				

we assume that 2 particles are required for infection the agreement is much worse, the value for chi square being 10 times as great.

This experiment confirmed the opinion that the statistical method outlined could be used for determining the virulence of vaccine virus. Therefore, since we possessed a method for determining the number of virus particles which are probably required to cause infection, we decided to apply it to other strains. The cultured strain of virus was studied next.

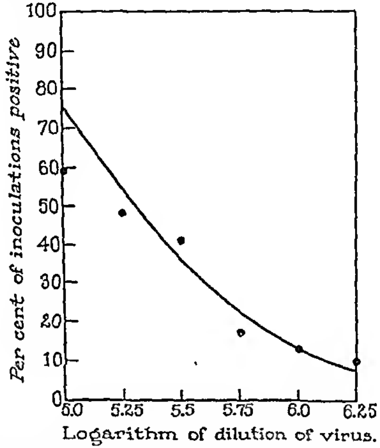

FIG. 3. Titration of cultured vaccine virus. Solid line represents theoretical curve, calculated on hypothesis that a single particle causes infection. Dots are experimentally determined points.

Experiment 2.—A suspension of cultured virus was prepared as follows: Chick embryo-Tyrode solution medium contained in several flasks was inoculated with virus. After 5 days the contents of all the flasks was pooled and the mixture centrifuged for 10 minutes at 1500 R.P.M. in order to throw down the tissue fragments. The sediment was removed from the tube, ground with sand, and the supernatant fluid from centrifugation was mixed with the ground sediment. The reconstituted virus suspension was then centrifuged at 3000 R.P.M. for 15 minutes in the horizontal centrifuge, the supernatant fluid pipetted off and again centrifuged at 3000 R.P.M. for 15 minutes. This was done in order to remove, as far as

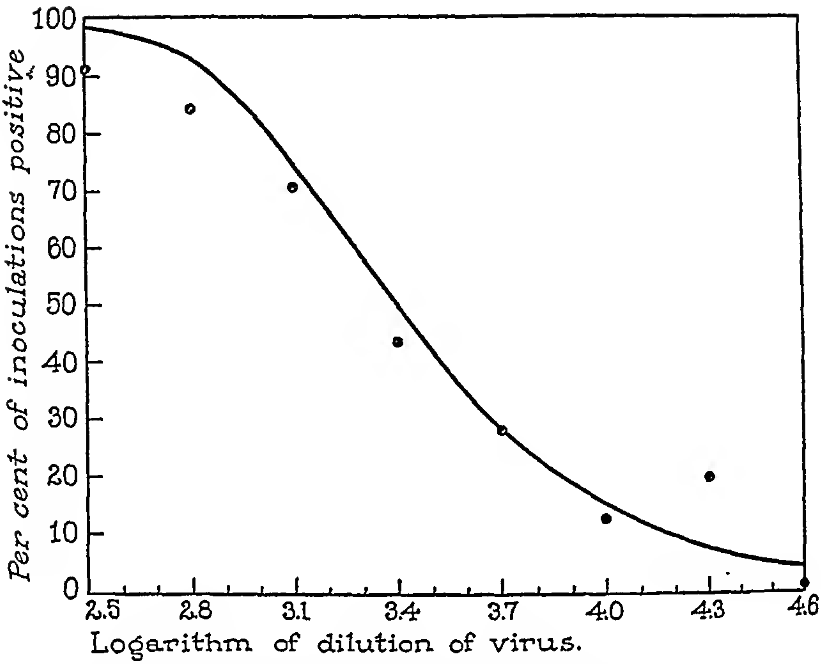

FIG. 4. Titration of cultured vaccine virus. Solid line represents theoretical curve, calculated on hypothesis that a single particle causes infection. Dots are experimentally determined points.

TABLE II

Experiment 2. Titration of Cultured Vaccine Virus. Calculations of Goodness of Fit of Experimental and Theoretical Curves

Logarithm of virus dilution	Results of experiment		If 1 particle initiates infection		If 2 particles initiate infection		If 3 particles initiate infection	
	Number of inoculations	Number positive (x)	Number expected positive ($\bar{x}$)	$\frac{(x-\bar{x})^2}{\bar{x}}$	Number expected positive	$\frac{(x-\bar{x})^2}{\bar{x}}$	Number expected positive	$\frac{(x-\bar{x})^2}{\bar{x}}$
5.00	22	13	15.7	0.47	19.2	2.00	19.8	2.36
5.25	27	13	14.7	0.20	15.1	0.29	15.6	0.43
5.50	27	11	9.7	0.18	8.6	0.67	6.6	2.94
5.75	29	5	6.4	0.31	5.2	0.01	2.3	1.26
6.00	30	4	3.9	0.03	1.5	4.17	0.6	19.30
6.25	30	3	2.3	0.21	0.6	9.58	0.3	24.40
			χ^2	1.40		16.72		50.69
			P	0.93				

TABLE III

Experiment 3. Titration of Cultured Vaccine Virus. Calculations of Goodness of Fit of Experimental and Theoretical Curves

Logarithm of virus dilution	Results of experiment		If 1 particle initiates infection		If 2 particles initiate infection	
	Number of inoculations	Number positive (x)	Number expected positive ($\bar{x}$)	$\frac{(x-\bar{x})^2}{\bar{x}}$	Number expected positive	$\frac{(x-\bar{x})^2}{\bar{x}}$
2.5	32	29	31.9	0.27	32.0	0.28
2.8	32	27	30.2	0.34	31.9	0.56
3.1	32	23	24.0	0.04	26.3	0.42
3.4	32	14	16.0	0.25	16.0	0.25
3.7	31	9	9.0	0.00	6.5	0.96
4.0	36	5	5.8	0.11	2.5	2.50
4.3	36	8	2.9	9.00	0.7	75.62
4.6	36	0	1.4	1.96	0.2	0.04
			χ^2	11.97		80.63
			P	0.10		

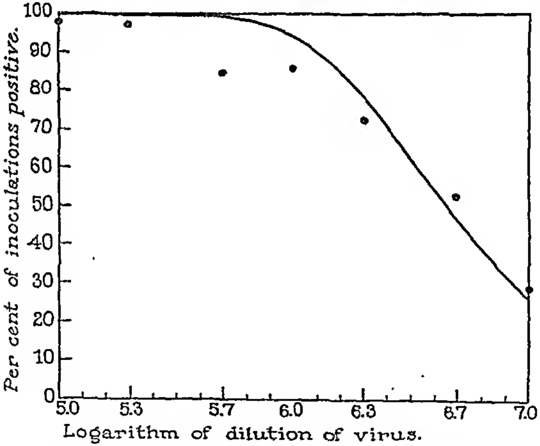

Fig. 5. Titration of Noguchi strain of vaccine virus. Solid line represents theoretical curve, calculated on hypothesis that a single particle causes infection. Dots are experimentally determined points.

possible, tissue debris and clumps of elementary bodies. An accurate titration of this virus suspension was carried out in the manner described in Experiment 1.

Experiment 3.—A second experiment with cultured virus was done, with a suspension prepared in a different manner. After centrifuging the virus culture in the horizontal centrifuge at 3000 R.P.M. for 15 minutes to remove debris, the virus particles remaining in the supernatant fluid were sedimented in the angle centrifuge, and then resuspended in buffer solution. This suspension was then subjected to two 15 minute periods of centrifugation in the horizontal centrifuge, the supernatant fluid being saved each time. It was then titrated in the manner which has been described.

TABLE IV

Experiment 4. Titration of Noguchi Strain of Vaccine Virus. Calculation of Goodness of Fit of Experimental and Theoretical Curves

Logarithm of virus dilution	Results of experiment		If 1 particle initiates infection		If 2 particles initiate infection		If 3 particles initiate infection	
	Number of inoculations	Number positive (x)	Number expected positive ($\bar{x}$)	$\frac{(x-\bar{x})^2}{\bar{x}}$	Number expected positive	$\frac{(x-\bar{x})^2}{\bar{x}}$	Number expected positive	$\frac{(x-\bar{x})^2}{\bar{x}}$
5.0	32	31	32.0	0.031	32.0	0.031	32.0	0.031
5.3	32	31	32.0	0.031	32.0	0.031	32.0	0.031
5.7	32	27	32.0	0.785	32.0	0.781	32.0	0.781
6.0	36	31	34.2	0.095	36.0	0.695	36.0	0.695
6.3	33	24	26.1	0.170	30.4	1.350	32.4	2.190
6.7	32	17	14.7	0.360	16.0	0.063	16.0	0.063
7.0	40	11	10.6	0.015	8.4	0.806	6.8	2.60
			χ^2	1.48		3.71		6.59
			P	0.96		0.70		0.40

The results of titration of these two samples of cultured vaccine virus are portrayed in Figs. 3 and 4. The data and calculations of goodness of fit of the titration curves are given in Tables II and III. It is seen that the results of the 2 experiments are essentially similar, and that the curves agree well with the calculated ones although the agreement is not quite so close as is the case with the Board of Health strain. This is probably due to two factors, *viz.*, greater difficulty in classifying the results of inoculations in a few instances, and the use of a smaller number of animals with a consequent increase in the standard deviation of each determination. Nevertheless, it is evident that by far the best fit is obtained by using the curve calculated for 1 or more particles causing infection.

A final experiment was performed using the Noguchi strain of virus.

The virus was used in the form of a suspeosion of elemeotary bodies, which was prepared io the usual way from the rabbit dermal eruption. The final suspeosion was twice centrifuged in the horizontal centrifuge in order to ensure that only discrete particles remaioed in suspension. An exact titration of the virus was carried out as in the precediog experiments, after determining the approximate iofective titer of the suspensioo.

The results of this experiment are portrayed in Fig. 5, and the calculations presented in Table IV. Again it is seen that the best agreement obtains between the experimental curve and the curve postulated on the basis that a single particle gives rise to infection.

DISCUSSION

Several of the viruses infecting animals are distributed in suspension in particulate form, and some of them appear to be associated with elementary bodies, which under proper conditions can be made visible with the microscope. A group of related problems at once present themselves. Is 1 of these particles, properly introduced into the tissues of the host, capable of setting up infection? Or is it necessary, in order to overcome the natural resistance of the host to infection, to introduce several of the particles? Do highly virulent invasive strains of virus differ from less virulent ones in the number of virus particles which are required in order to initiate infection? The experiments described were undertaken to answer these questions as applied to the virus of vaccinia.

Aside from their application in studies of virulence of the virus of vaccinia, the results of the experiments suggest important considerations with regard to the titration of virus suspensions. It is evident that if a single virus particle gives rise to infection, the determination of the number of particles present in a suspension by the inoculation of a single series of serial tenfold dilutions into animals will be attended by a very large experimental error. In order to determine more accurately the number of infectious particles present it is necessary to perform several series of inoculations of the suspension, which has been suitably diluted. The results must then be subjected to some kind of statistical analysis. Three methods for doing this are

available. (*a*) Several series of inoculations may be performed and each series regarded as an individual titration. The end point of each may be recorded and an arithmetic mean of the end points calculated. Accuracy is increased but in a small series a single result differing widely from the mean will unduly influence the result. The difficulty is not solved by discarding the discrepant result and taking the "2 results which agree." (*b*) If infection can be shown to follow inoculation of a single particle, tables of probability can be set up, so that from the results of a series of inoculations the most probable concentration of infectious particles in the original suspension can be derived. The use of that method is greatly facilitated by the extensive tables provided by Halvorson and Ziegler (15). (*c*) The dilution of suspension may be determined, which would be expected on

TABLE V

Calculation of 50 Per Cent End Point in Titration of Virus

Logarithm of virus dilution	Inoculations positive	Inoculations negative	Accumulation, positive	Accumulation, negative	Positive
					per cent
3	4	0	12	0	100
4	4	0	8	0	100
5	3	1	4	1	80
6	1	3	1	4	20
7	0	4	0	8	0

inoculation to give rise to an equal number of positive and negative results (16). A simple method for calculating this[2] was given in our previous paper (7).[3] Of the 3 methods of reduction of data, the third

[2] Suggested to us by Dr. Muench.

[3] This method of computing the strength of a virus suspension has been in use for several years in the Yellow Fever Laboratories of the International Health Division of The Rockefeller Foundation. Its use was described in a previous paper (7), but for convenience will again be described here.

A series of tenfold dilutions of the infective agent is prepared, and a number (at least 4 and preferably a larger number) of inoculations are made of each dilution. The results are recorded as in columns 2 and 3 of Table V. (This is the protocol of an actual titration.) Then the positive and negative results, respectively, are accumulated, the direction in which the accumulation is made being of primary importance. Each column is added, beginning with the smaller end. The various

appears to us to be the best. It is based on sound principles, is easy of performance, and yields an accurate estimate of the concentration of infectious particles. Since it presumes only that the titration curve is a symmetrical one, it does not require for its use the assumption that a single particle gives rise to infection, a prerequisite to the use of the second method. If, however, it is known that a single particle will infect, the exact number of infectious particles per unit volume may be derived easily from the 50 per cent end point should that information be desired.

SUMMARY

A method has been described by which it is possible to estimate the number of particles of vaccine virus which are required to cause infection in the rabbit skin. The method consists essentially in performing a series of intradermal inoculations in rabbits of suitably diluted virus suspensions. The percentage of inoculations at each dilution giving rise to lesions is observed, and the data are subjected to appropriate statistical analysis. Several strains of vaccine virus, differing in their characteristics, have been studied with the following results. Infection with the New York City Board of Health virus appears to follow the injection of a single particle of virus. The same is true for the strain derived from it but cultured in a chick embryo-Tyrode solution medium for a prolonged period. This strain, as has been noted, has largely lost its ability to cause extensive necrosis in the rabbit skin, and causes generalized infection only exceptionally. From the results here reported, it appears that other factors are responsible for the altered character of the lesion than the ability of the virus to establish a foothold in the animal organism. In this respect the cultured appears to be the equal of the original passage virus. Similarly the Noguchi strain of virus is apparently capable of infecting, if a single virus particle is properly introduced.

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sums in each column then represent the number of inoculations positive at that dilution and higher dilutions, or the number negative at that dilution and lower dilutions (columns 4 and 5). The percentage of positives at each dilution and higher dilutions is then calculated from the data of the summary columns (column 6). In this case there are 80 per cent positive reactions at 10^{-5} and 20 per cent at 10^{-6}. 50 per cent then would be 30/60 or 0.5 of the distance from 10^{-5} to 10^{-6} or $10^{-5.5}$.

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SEROLOGICAL EVIDENCE FOR THE OCCURRENCE OF INFECTION WITH HUMAN INFLUENZA VIRUS IN SWINE

By RICHARD E. SHOPE, M.D.

(From the Department of Animal and Plant Pathology of The Rockefeller Institute for Medical Research, Princeton, New Jersey)

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Elkeles (1) and Shope and Francis (2) demonstrated that swine could be infected experimentally with human influenza virus (3). The disease resulting was extremely mild and was similar clinically and at autopsy to that observed in swine infected with swine influenza virus alone (4). When small amounts of a culture of *Hemophilus influenzae suis* (5) were administered with the human virus, a more prostrating febrile illness, similar to true swine influenza although never so severe, usually resulted. Furthermore, the disease induced in swine by the human influenza virus could be transmitted only rarely to normal swine by exposure (2), whereas swine influenza is highly contagious (6). Because of this, the opinion was expressed that it seemed unlikely that the current human influenza virus could become established in swine under field conditions and progress as the cause of any widespread swine disease (2). Within the past year, however, two swine herds that have been under study have furnished evidence to indicate that this opinion may have been at least partially wrong. It is the purpose of this paper to report the findings which indicate that, in these two herds, infection with human influenza virus actually occurred under field conditions as they prevail on eastern farms.

History of Swine Herds Studied

1. Bordentown.[1]—On May 24, 1937, two sick swine from the New Jersey State Prison Farm at Bordentown were brought to the laboratory for diagnosis. The

[1] We are indebted to Mr. J. S. Karlberg, Dr. Howard Wiesler, and Mr. John Grehan for their cooperation in the collection of material and information at the Bordentown farm.

autopsy findings were those of hog cholera. Blood serum from one of the animals was tested for the presence of neutralizing antibodies against the pseudorabies and the human and swine influenza viruses. It failed to neutralize swine influenza or pseudorabies virus but did neutralize human influenza virus. This finding was surprising and entirely unexpected. Sera of swine from a number of sources had in the past been tested against human influenza virus, and neutralizing antibodies had never before been encountered. It was known, however, from earlier work (2) that the sera of swine recovered from experimental infection with human influenza virus contained human virus-neutralizing antibodies. This suggested strongly that the Bordentown pig whose serum neutralized human influenza virus had undergone an earlier human influenza virus infection. A further study of the herd at Bordentown was therefore undertaken.

The farm was visited and the man in charge of the herd interviewed. Only 7 swine remained alive in the pen from which the 2 original sick animals had been taken. However, a nearby pen contained approximately 80 healthy swine of the same general age. The herdsman stated that he had observed no sick pigs in either group prior to the onset of the hog cholera outbreak in the one pen. Blood was obtained by tail bleeding from 6 of the 80 normal swine and from the 7 sick animals. Sera from these 13 pigs were tested for neutralizing antibodies against the swine and human influenza viruses. All 13 sera neutralized human influenza virus; none neutralized the swine influenza virus. These results constituted good evidence that the herd under study had undergone an earlier infection with human influenza virus and that few if any of the animals had escaped infection. The fact that sera from the normal as well as the sick swine contained neutralizing antibodies indicated that the hog cholera outbreak in May was in all probability totally unrelated to and in no way the result of the unrecognized human influenza virus infection.

All of the swine on the farm had been born prior to November, 1936, and had thus lived through a winter during which epidemic influenza was known to have been prevalent (7, 8). In order to obtain sera for control purposes from swine on the same farm, but from animals born long after influenza may have been present in the prison farm human population, further studies were postponed until October and November, 1937. By this time the fall pigs could be bled for control sera. Blood was obtained by tail bleeding from 8 of these young pigs born after July, 1937, and by throat bleeding at slaughter from 15 more of the old hogs born prior to November, 1936. These sera were all tested for neutralizing antibodies against the human and swine influenza viruses. The results obtained, together with those obtained with the sera drawn in May and June, will be outlined subsequently in Table I.

2. *Jamesburg.*[2]—The swine herd on the farm of the New Jersey State Home for Boys at Jamesburg contained, when first seen in November, something over

[2] We are indebted to Mr. William Mills for his cooperation in the collection of material and information at the Jamesburg farm.

100 hogs over 1 year of age, weighing from 200 to 300 pounds apiece, and about the same number of small pigs born during the autumn of 1937. All animals appeared in fine physical condition nor was there any history of past illness. Blood was obtained by tail bleeding from 7 of the small pigs and by throat bleeding at slaughter from 20 of the old hogs. The sera thus obtained were tested for neutralizing antibodies against the human and swine influenza viruses and the results will be outlined subsequently in Table II.

Recent Respiratory Tract Disease History of Human Populations from Which the Swine Herds Could Have Acquired Human Influenza Virus

1. Bordentown.—The garbage fed to the swine at Bordentown came from the dining rooms of the prison at Trenton and the prison farm at Bordentown, and the swine were tended by prison inmates. Dr. Howard Wiesler, resident physician, found on examining his records, that he had seen some 45 respiratory tract conditions among the Bordentown inmates during December, 1936, and January, 1937. Most of these cases were simple coryzas. Only 4 had been febrile and ill enough to go to bed. One of these 4 febrile cases was, at the time of his illness, assigned to work at the pig lots. He reported sick on January 19, 1937, and remained in bed for 3 days. At the Trenton prison during the same period, there were 3 cases that were clinically suggestive of influenza, and 2 pneumonias. The incidence of upper respiratory tract ailments and of influenza in the Trenton district as a whole was above average during the corresponding period.

2. Jamesburg.—The garbage fed the swine at Jamesburg came from the dining rooms of the New Jersey State Home for Boys and the swine were tended by inmates of the institution. Fortunately, so far as the present experiments are concerned, a definite history of influenza among the inmates of the institution is furnished by the studies of Stokes, McGuinness, Langner, and Shaw (8) conducted during the fall and winter of 1936–37. Among a population of 550 inmates they record the occurrence of 219 cases of upper respiratory tract disease. 55 of these cases were febrile, and 164, afebrile. Influenza virus, typical in all respects, was isolated, by ferret inoculation, from one of the febrile cases.[3]

EXPERIMENTAL

The neutralization tests were conducted in white mice, using a technique previously described (9). The human influenza viruses used were Francis' PR 8 strain (10) and the P-37-9 strain of Stokes and his coworkers (8). The swine influenza virus employed was strain

[3] We are indebted to Stokes and his coworkers for furnishing a mouse-adapted strain of this Jamesburg virus (strain P-37-9) for use in studies of the neutralizing antibodies in sera from the Jamesburg swine herd.

15 (Iowa, 1930). All three viruses had been well adapted to white mice and regularly killed these animals in the dosages used in the present experiments.

The supernatant of a 2 per cent suspension of infected mouse lung was employed as virus in the cases of the PR 8 strain human virus and the strain 15 swine virus, and of a 5 per cent suspension in the case of the P-37-9 strain. Virus was mixed in equal parts with the undiluted sera to be tested, and the mixtures stored for 2 hours in the refrigerator prior to their administration to the test mice. 3 etherized mice were inoculated in each test by dipping their noses in the virus-serum mixture contained in a slightly tilted small Petri dish. Surviving mice were killed on the 7th day and their lungs, together with those of mice dying earlier, were examined for the presence of influenza lesions (10–12). Mice which succumbed during the 7 day observation period and showed typical influenza pulmonary pathology at autopsy were considered to have received a non-neutralizing serum. Those which survived the 7 day period were considered to have received a neutralizing serum. The 7 day period of observation was chosen because experience has shown that, with the dosage of virus employed, no further deaths occur after that period. Within individual groups, where results were split and only one or two of the mice died, judgment of the neutralizing capacity of the serum under test was determined by the survival or death of the majority of the mice in the group. Usually, however, the results obtained were clear cut and all mice in the group either died or survived.

RESULTS

1. Bordentown Swine.—The results obtained with the sera from the Bordentown farm swine are outlined in Table I.

As shown in Table I, the sera of none of the swine born after July, 1937, neutralized the human influenza virus, whereas the sera of all 29 of those born prior to November, 1936, did neutralize. Roughly half the sera of these older animals not only prevented death but neutralized the virus so completely that lung lesions were not encountered in the surviving test mice when these were autopsied on the 7th day.

While there had been no history of a swine influenza infection in the Bordentown herd during the winter of 1936–37, the sera obtained were all tested for their capacity to neutralize swine influenza virus in order to eliminate from consideration the possibility that the human influenza virus-neutralizing antibodies might really represent cross-neutralizing antibodies resulting from an earlier swine influenza infection. As shown in Table I, the sera of none of the Bordentown

TABLE I

Neutralization Tests against the Viruses of Swine and Human Influenza with Sera from Swine on New Jersey State Prison Farm, Bordentown, New Jersey

Serum from swine No.	Serum tested for capacity to neutralize					
	PR 8 strain human influenza virus			Strain 15 swine influenza virus		
	Mouse No.			Mouse No.		
	I	II	III	I	II	III
(*a*) Swine born after July, 1937						
T- 1	D 4*	D 4	D 4	D 4	D 4	D 4
T- 2	D 4	D 5	D 5	D 4	D 4	D 4
T- 3	D 2	D 3	D 7	D 5	D 5	D 5
T- 4	D 2	D 2	D 4	D 3	D 4	D 4
T- 5	D 2	D 2	D 6	D 4	D 4	D 4
T- 6	D 2	D 5	D 5	D 3	D 3	D 3
T- 7	D 4	D 6	D 6	D 4	D 4	D 4
T- 8	D 2	D 4	D 6	D 3	D 3	D 3
(*b*) Swine born before November, 1936						
S- 1	S	S	S	D 4	D 4	D 4
S- 2	S	S	S	D 4	D 5	D 5
S- 3	S	S	S	D 6	D 6	D 7
S- 4	S	S	S	D 5	D 5	D 6
S- 5	D 6	S	S	D 3	D 4	D 4
S- 6	S	S	S	D 4	D 6	S
S- 7	S	S	S	D 4	D 5	D 6
S- 8	S	S	S	D 5	D 5	D 6
N- 1	S	S	S	D 5	D 5	D 5
N- 2	S	S	S	D 5	D 5	D 6
N- 3	S	S	S	D 2	D 4	D 7
N- 4	S	S	S	D 2	D 4	D 4
N- 5	S	S	S	D 5	D 5	S
N- 6	S	S	S	D 4	D 4	D 4
N- 7	S	S	S	D 5	D 6	D 6
N- 8	S	S	S	D 3	D 3	D 3
N- 9	S	S	S	D 4	D 5	D 7
N-10	S	S	S	D 4	D 5	D 6
N-11	S	S	S	D 4	D 5	D 6
N-12	S	S	S	D 4	D 4	D 4
N-13	S	S	S	D 6	D 6	D 7
N-14	S	S	S	D 5	D 6	D 6
N-15	S	S	S	D 4	D 5	D 6
N-16	S	S	S	D 5	D 6	D 6
N-17	S	S	S	D 7	D 7	S
N-18	S	S	S	D 6	D 6	D 7
N-19	S	S	S	D 6	D 7	S
N-20	S	S	S	D 4	D 4	D 4
N-21	S	S	S	D 3	D 4	D 5

* D 4 = died on 4th day.

S = survived. (Experiment terminated on 7th day.)

TABLE II

Neutralization Tests against the Viruses of Swine and Human Influenza with Sera from Swine on Farm of New Jersey State Home for Boys, Jamesburg, New Jersey

Serum from swine No.	Serum tested for capacity to neutralize								
	P-37-9 strain human influenza virus			PR 8 strain human influenza virus			Strain 15 swine influenza virus		
	Mouse No.			Mouse No.			Mouse No.		
	I	II	III	I	II	III	I	II	III
(*a*) Swine born after July, 1937									
YS 1	D 4*	D 5	D 6	D 3	D 4	D 4	D 3	D 3	
YS 2	D 4	D 5	D 5	D 4	D 4	D 5	D 3	D 4	D 4
YS 3	D 3	D 4	D 4	D 3	D 4	D 4	D 3	D 3	D 4
YS 4	D 4	D 5	D 6	D 6	D 6	D 7	D 3	D 3	D 4
YS 5	D 4	D 5	D 5	D 4	D 6	D 6	D 3	D 3	D 4
YS 6	D 4	D 4	D 4	D 3	D 4	D 4	D 3	D 3	D 4
YS 7	D 3	D 3	D 4	D 4	D 4	D 6	D 3	D 3	D 3
(*b*) Swine born before November, 1936									
OS 4	D 6	D 7	S	D 6	D 7	D 7	D 3	D 4	D 4
OS 6	D 6	D 6	D 6	D 5	D 6	D 6	D 3	D 3	D 4
OS 7	S	S	S	D 4	D 4	D 5	D 4	D 4	D 4
OS 9	D 5	D 5	D 5	D 5	D 5	D 5	D 3	D 3	D 3
OS 15	D 4	D 5	D 6	D 3	D 4	D 4	D 3	D 3	D 4
OS 1	S	S	S	S	S	S	D 3	D 3	D 3
OS 2	S	S	S	S	S	S	D 3	D 4	D 5
OS 3	S	S	S	S	S	S	D 3	D 4	D 4
OS 5	S	S	S	S	S	S	D 3	D 4	D 4
OS 8	S	S	S	S	S	S	D 4	D 4	D 4
OS 10	S	S	S	S	S	S	D 3	D 3	D 3
OS 11	S	S	S	S	S	S	D 3	D 4	D 5
OS 12	S	S	S	S	S	S	D 4	D 4	S
OS 13	S	S	S	S	S	S	D 4	D 4	D 5
OS 14	S	S	S	S	S	S	D 3	D 3	D 3
OS 16	S	S	S	S	S	S	D 3	D 4	D 5
OS 17	S	S	S	S	S	S	D 5	D 6	D 6
OS 18	S	S	S	S	S	S	D 2	D 5	S
OS 19	S	S	S	S	S	S	D 3	D 3	D 4
OS 20	S	S	S	S	S		D 3	D 4	D 5

* D 4 = died on 4th day.
S = survived. (Experiment terminated on 7th day.)

swine, either old or young, contained neutralizing antibodies for swine influenza virus. The swine had thus clearly not undergone a previous infection with swine influenza, and the neutralizing antibodies for human influenza virus encountered in the sera of the older animals had not resulted from such infection.

2. *Jamesburg Swine.*—The sera from the swine on the Jamesburg farm were tested for their capacity to neutralize, not only the PR 8 strain and strain 15 viruses, but the P-37-9 human strain as well, since this virus had originally been recovered from a case of influenza occurring in one of the institution inmates. It was conceivable that the P-37-9 virus might be more appropriate to use, under the circumstances, than the PR 8 strain. The results obtained are given in Table II.

As shown in Table II, the sera of none of the 7 young swine neutralized either strain of human influenza virus. However, the sera from 16 of the old hogs neutralized the P-37-9 strain human virus while the sera of 15 neutralized the PR 8 strain. One serum sample from an old hog (OS 7) neutralized the P-37-9 strain very effectively but failed to neutralize the PR 8 strain. Repeated tests of this serum against the two human viruses have confirmed the correctness of this result. While roughly half of the Bordentown swine sera had neutralized the human virus so thoroughly that lung lesions were completely suppressed in the test mice, none of the Jamesburg sera achieved such solid protection against the PR 8 strain. However, against the P-37-9 strain, 5 of the 16 neutralizing sera neutralized so completely that no lung lesions were encountered in the surviving test mice when these were autopsied at the end of the experiment.

None of the Jamesburg sera neutralized swine influenza virus, indicating that, as in the case of the Bordentown herd, the human influenza virus-neutralizing antibodies had not resulted from earlier infection of the herd with swine influenza.

DISCUSSION

The sera from two age groups of swine on two New Jersey institution farms have been studied for their capacity to neutralize the swine and human influenza viruses. The sera from none of the young swine, born since July, 1937, neutralized either virus. However, the sera

of all of the old hogs studied on one farm and of three-fourths of those studied on the other farm neutralized human influenza virus, although failing to neutralize swine influenza virus. These older animals had all been born prior to November, 1936, and had thus lived through a winter when human influenza was known to have been unusually prevalent. The presence of human influenza virus-neutralizing antibodies in the sera of the older animals was not an age phenomenon, because sera from swine of corresponding ages from other sources have failed to neutralize human influenza virus. It is believed, on the basis of the known behavior of swine to experimental infection, that the antibodies in the sera of the older animals resulted from actual infection with human influenza virus and that, in both herds studied, a widespread infection of human origin had occurred. The failure to recognize either outbreak is not surprising because, even under conditions of experimental infection of swine with large doses of human influenza virus alone, the resulting disease is so mild and ill defined as to be difficult of certain recognition (1, 2). How the virus was transferred to swine is unknown, though presumably it was either by direct exposure to human cases or through the medium of garbage contaminated by virus. In either event the initial infection must have been so extensive as to involve all of the swine on the Bordentown farm and three-fourths of those on the Jamesburg farm, unless human influenza virus infection of swine under farmyard conditions is more highly contagious than it could be shown to be in the laboratory (2).

One apparent discrepancy in the results obtained with the Jamesburg sera deserves comment. The serum from swine OS 7 neutralized the P-37-9 strain human influenza virus but failed to neutralize the PR 8 strain. Certain antigenic differences between various strains of human influenza virus have recently been observed (13, 14) and it seems possible that the discrepancy with the OS 7 serum may indicate that more than one strain of virus was prevalent in the human population at Jamesburg and that more than the one strain was transmitted to the swine. The same suggestion is afforded by the results with the Bordentown sera. Here roughly half of the sera neutralized the PR 8 virus completely, as evidenced by protection of the test mice not only

against death but against the development of any lung lesions as well. The other half of the sera protected against death but did permit the development, in the lungs of the test mice, of a varying amount of influenza virus pneumonia. While these differences may have been dependent solely upon quantitative differences in the amounts of antibody contained by the sera, they may equally well reflect differences in the antigenic structure of the viruses responsible for their generation. Thus one virus may have been of an antigenic type very similar to the PR 8 strain and have caused the production of antibodies that completely neutralized the PR 8 virus while the other virus may have been of a slightly different antigenic type and have produced antibodies only partially neutralizing the PR 8 strain. Whatever the antigenic compositions of the influenza viruses infecting the swine at Bordentown and Jamesburg may have been, they were definitely and quite completely different from that of ordinary swine influenza virus.

Previous to the experiments just described there has been no concrete evidence that influenza virus could be transmitted from man to swine under natural conditions. As long ago as 1918, however, there was, in the Middle West, the popular belief, first voiced by Dr. J. S. Koen, that swine could acquire influenza from man and that swine influenza had had its origin from man during the 1918 pandemic (15, 16). Numerous similarities between the viruses of swine and human influenza, together with the history that swine influenza appeared for the first time during the 1918 human pandemic, led Laidlaw to propound the theory that swine influenza virus represented a surviving form or prototype of the 1918 pandemic human virus (17); a theory to which we subscribed (18). The present experiments, by demonstrating that human influenza virus of the type prevalent during the winter of 1936–37 was transmitted to swine under natural conditions, furnish evidence that a similar transmission from man to swine might readily have occurred in 1918. The failure of recent strains of human influenza virus to cause widely disseminated porcine epizootics like those caused annually in the Middle West by swine influenza virus may be explained by the low contagiousness, when in swine, of the current human influenza viruses (2).

SUMMARY

Antibodies capable of neutralizing human influenza virus were present in the sera of old swine on two New Jersey institution farms, but absent from the sera of young swine on the same farms. The old animals had lived through the winter of 1936–37 in which outbreaks of upper respiratory tract disease were prevalent among the human inmates of the two institutions, while the young swine studied were born long after these outbreaks. It is believed that the swine whose sera neutralized human influenza virus had undergone an unrecognized human influenza virus infection acquired from man. The possible bearing of these observations upon the theory that swine influenza was originally of human origin is discussed.

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THE EXCRETION OF CYANOL, AZOFUCHSIN I AND WATER BY THE KIDNEYS OF RABBITS*

By WILLIAM E. EHRICH, M.D., GEORGE M. BARTOL, AND RICHARD E. WOLF

(From the Department of Pathology, University of Pennsylvania, School of Medicine, Philadelphia)

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In renal disorders function and morphology have not yet been correlated satisfactorily. It is true that the fundamentals have been clarified by the work of Volhard and Fahr; but when we come to details, our knowledge is still incomplete. As recent advances, both in the understanding of renal function and in the experimental production of renal diseases, justified a new attempt in this direction, certain renal disorders have been reproduced, and a comparison of the morphological changes with the changes in function has been made.

It may be regarded as definitely established that certain substances are eliminated by the kidneys solely or mainly by filtration through the glomeruli, while others are mainly secreted by the tubules. Therefore, in the present experiments we studied the elimination by the kidneys: (*a*) of water; (*b*) of a dye generally regarded as eliminated by glomerular filtration; and (*c*) of a dye believed to be secreted by the tubular epithelium. While it has often been said that dye tests are of no great value, more recent studies have shown that a properly applied phenol red test, for example, is at least as useful as the urea clearance test (Shaw, 1925; Ockerblad, 1928; Chisholm, 1930; Chapman and Halsted, 1933). Watanabe, Oliver and Addis (1918) appear to have been right when they pointed out that these tests in reality give more than either rate of excretion or blood concentration alone. If the same amount of dye always is injected, the

* Presented in brief at the Scientific Exhibition of the American Medical Association at Atlantic City, June, 1937, and at the Scientific Exhibition of the Tenth Annual Graduate Fortnight of the New York Academy of Medicine, November, 1937.

plasma concentration tends to be constant, and thus the ratio between the amount of the dye in the blood and in the urine, or in other words, the clearance, can be obtained.

For a "glomerular" dye, cyanol was chosen; and for a "tubular" dye we at first used phenol red. As the latter is an indicator, we found it difficult, however, to determine its concentration when the urine was rich in phosphates, or when the concentration of the dye was low: therefore azofuchsin I[1] was finally selected. As little was known about the excretion of these dyes in rabbits, we first had to study their normal elimination. The results of this study are the subject of this paper.

LITERATURE

Cyanol was first studied by Hoeber and his pupils. According to Yoshida (1924) and Orzechowski (1930), it is a highly diffusible, lipoid insoluble, acid dyestuff which easily diffuses through parchment cylinders, but does not enter erythrocytes or the lipoid mixture of Nirenstein. They found that in double perfusion experiments with a modified Ringer solution, in frogs, the dye either did not appear at all, or scarcely appeared, in the urine, when offered to the tubules alone. However, if it was offered to the glomeruli only, or to both glomeruli and tubules, it was concentrated one to two times (Yoshida, 1924; Schulten, 1925; Hoeber, 1927; Scheminzky, 1929; Robbins and Wilhelm, 1933).

Studying the kidney of aglomerular toadfish, Hoeber (1930) was unable to detect any dye in the urine. Marshall and Grafflin (1932) were also unable to find any dye after injections of 10 to 15 mg. per kilo. However, if they injected such tremendous doses as 125 to 300 mg. per kilo, they found a small amount in the urine. This latter finding was later confirmed by Grafflin (1936).

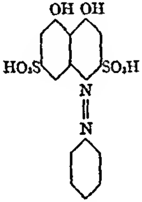
In glomerular sculpins which had been rendered functionally aglomerular by two or more doses of 200 to 300 mg. of phlorizin per kilo, Marshall and Grafflin (1932) found no excretion of cyanol, whereas normal sculpins excreted the dye and concentrated it 1.3 to 2.4 times (Grafflin, 1936). In two phlorizinized sculpins Grafflin found the cyanol clearance to be but 67 to 77 per cent of the simultaneous glucose clearance. However, both these fish had received tremendous doses of cyanol.

In rabbit urine Steffanutti (1930) found phenol red nine times as concentrated as cyanol, both dyes having been given simultaneously. In a rabbit which had been poisoned with uranium the phenol red concentration amounted to but a third of the cyanol concentration. Cope (1934) who studied the cyanol clearance in

[1] For the choice and the supply of both the dyes we are indebted to Dr. R. Hoeber.

rabbits as compared with the xylose and sucrose clearances, found the sucrose clearance to be about 25 per cent higher than the cyanol and xylose clearances. In phlorizinized rabbits the glucose clearance rose to equal the xylose clearance, whereas the cyanol clearance fell to about 42 per cent of the two clearances. As from our experience, which conforms with that of Smith,[2] the cyanol figures of Cope appear to be much too high, and as the possibility cannot be excluded that the drop observed by this author may have been due to factors other than change in the renal elimination of the dye, we do not attach weight to this observation at present.

Azofuchsin I has been used by Hoeber and his pupils only (1930, 1932). Like cyanol it is a highly diffusible lipoid insoluble, acid dyestuff which does not permeate into erythrocytes, and which is practically insoluble in the lipoid mixture of Nirenstein. Its formula has been given as



In double perfusion experiments with a modified Ringer solution, in frogs, Orzechowski (1930) found it to be concentrated 11 to 65 times, if it was offered to the tubules only. *In excised frog kidneys which were placed in oxygenated salt solution containing azofuchsin, the dye behaved exactly like phenol red* (Hoeber and Meirowsky, 1932).

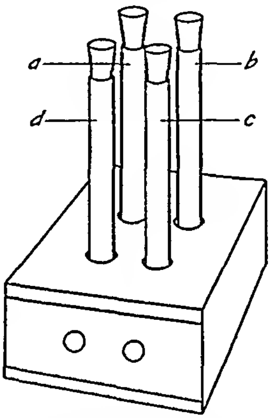
Method

Male Chinchilla rabbits weighing 1500 to 2200 gm. were used. Animals with evidence of spontaneous renal disease were discarded. At the beginning of an experiment each animal received 100 cc. of tap water by stomach tube. When the resulting diuresis was in progress, usually 1 hour after the introduction of the water, the dyes were injected intravenously. In all experiments 2 cc. of a 0.1 per cent solution were given. At first both dyes were given simultaneously, as done by Steffanutti (1930). But since the natural color of the urine changed a great deal during the experiments, we actually had to measure three colors in the same specimen. Since this could not be done with accuracy, we later injected but one dye at a time.

[2] Smith, Homer, oral communication.

The urine was collected by fine catheters which were inserted into the bladder immediately after the introduction of the water. The catheters held about 1.5 to 2.0 cc. of fluid. The volume of the urine was measured every 15 or 30 minutes, and after the injection of the dye, every 10 minutes. As a rule, urine passed freely when diuresis was in progress. If there seemed to be a retention of urine, the bladder was gently massaged each time urine was to be collected. A few experiments in which blood appeared in the urine were discarded.

The concentration of the dyes was tested with standard solutions which were prepared from the same solutions as those which were injected. To compensate for the varying color of the urine, it was found necessary to introduce into the comparator block a tube with urine of the same concentration as that of the specimen to be tested (Text-fig. 1). If the dye was concentrated very highly, it



TEXT-FIG. 1. Sketch of the comparator used. Tube (*a*) contained the urine specimen to be tested; (*b*) the standard dye solution in water; (*c*) the urine control which was diluted to the color of the urine to be tested, and (*d*) water. The front of the comparator was covered by milk glass.

was found to be more accurate to dilute the specimen with 4 or 9 parts of water before the determination. If the urine contained precipitated phosphate, this was dissolved by dilution with water, or if the dye concentration was low, by adding a trace of acid.

RESULTS

1. The excretion of *water* has been studied in 83 experiments (in 63 rabbits; 35 with cyanol, 38 with azofuchsin, 10 without dye). In 14 experiments in which 100 cc. of tap water was given by stomach tube on the day of the experiment ("dry" animals), the average rate of urine formation was 0.023 cc. per minute during the first 30 min-

utes, 0.063 cc. during the second, 0.197 cc. during the third, 0.223 cc. during the fourth and 0.273 cc. during the fifth period of 30 minutes. In 69 experiments in which the animals received 100 cc. of water the day before the experiment as well ("wet" animals), the corresponding figures were 0.018, 0.400, 0.447, 0.587 and 0.537 cc. per minute. The highest rate obtained amounted to 1.5 cc. per minute during a 10 minute period, or to 1.31 cc. during a period of 1 hour.

TABLE I

The Rate of Urine Excretion during the First 2½ Hours after the Ingestion of 100 Cc. of Water

	Rate of urine excretion per min.						
	0.02–0.07 cc.	0.08–0.2 cc.	0.2–0.4 cc.	0.4–0.6 cc.	0.6–0.8 cc.	0.8–1.0 cc.	1.0–1.15 cc.
"Dry" animals............	7*	2	3	2	0	0	0
"Wet" animals............	0	13	24	12	13	5	2

* Number of experiments.

TABLE II

The Peak of Urine Excretion after the Ingestion of 100 Cc. of Water

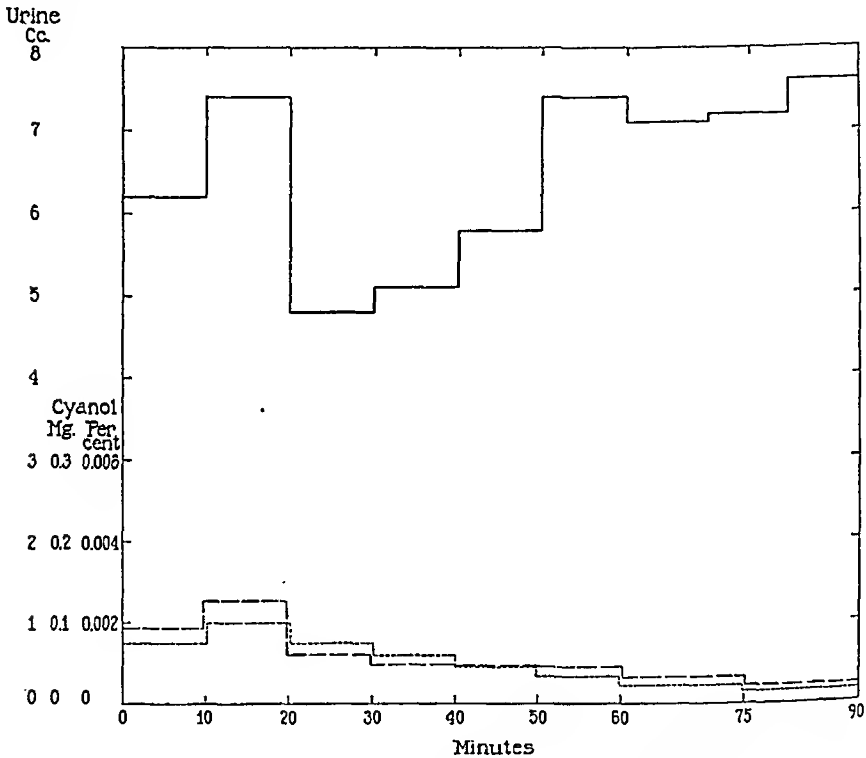
	Periods of 30 min.					
	1	2	3	4	5	6
"Dry" animals.............	0*	0	0	2	3	9
"Wet" animals.............	0	8	5	25	11–21†	10–20†

* Number of experiments.

† In 10 of these the experiment was stopped after the fifth period.

The individual rates varied a great deal (Table I). But whereas in most dry animals the rates were between 0.02 and 0.07 cc. per minute, in most wet animals they were between 0.08 and 0.8 cc. A similar variation was found in respect to the time the diuresis was at its peak (Table II). But whereas in the dry animals this was not reached before the fourth period and in the majority of the experiments at the sixth period only or later, in the wet animals it was reached as early as in the second period and in the majority of the experiments in the fourth or fifth period, *i.e.*, from 1½ to 2½ hours

after the ingestion of water. As these results are in close conformity with those of Rees (1918) and Heller and Smirk (1932), as far as the peak of the diuresis is concerned, and with those of Oehme (1921) and Walker, Schmidt, Elsom and Johnston (1937), as far as the difference between dry and wet animals is concerned, it may be taken for certain that under ordinary laboratory conditions a better water

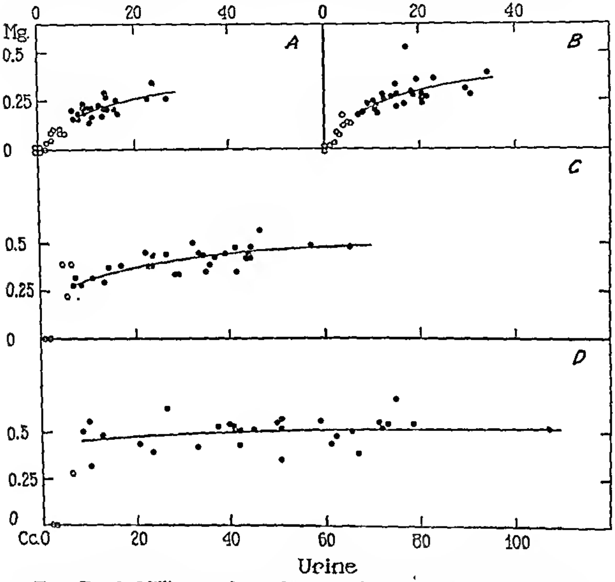


TEXT-FIG. 2. Average quantities of urine (———) and cyanol (mg. -----, concentration per cent ....) excreted in 10 minute periods after the intravenous injection of 2 mg. of the dye.

diuresis is obtained from rabbits, if they are given 100 cc. of water the day before the experiment as well as on the day of the experiment, and that under these conditions the diuresis is well in progress in the 2nd hour after the ingestion of water.

2. The excretion of *cyanol* has been tested in 35 experiments (in 30 rabbits). The highest rate of excretion, measured in mg., was

found to be in the first 10 minute period after the injection of the dye in 5 experiments, and in the second 10 minute period in 18 experiments. In all these experiments the rate of urine formation amounted to 0.35 cc. per minute or more during the first 20 minutes. In the remaining 12 experiments, in which the highest rate of dye



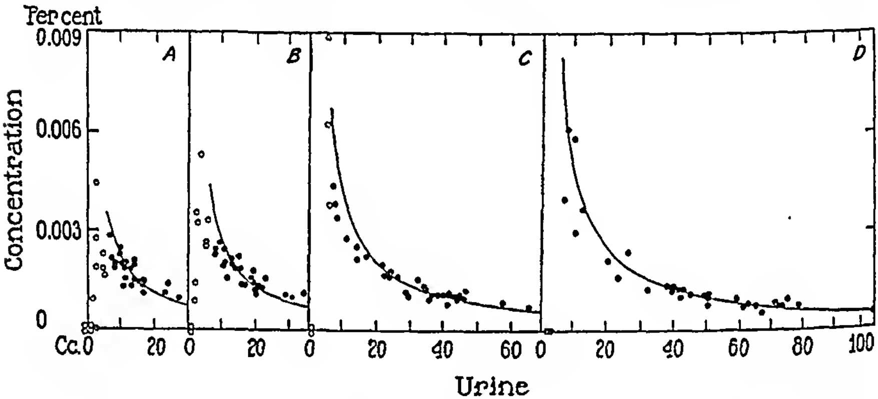
TEXT-FIG. 3. Milligrams of cyanol excreted during the first 20 (*a*), 30 (*b*), 60 (*c*) and 90 (*d*) minutes of the experiments as compared with the cc. of urine produced during these periods.

excretion was observed later than in the second 10 minute period, the rate of urine formation was 0.25 cc. per minute or less. The highest amount of dye excreted in a 10 minute period was 0.226 mg.; the highest concentration 0.017 per cent.

If we only take into account those experiments in which the urine

rate amounted to more than 0.25 cc. per minute during the first 20 minutes, the average rates of cyanol excretion during the first 9 periods follow the curve shown in Text-fig. 2. It should be noted that the urine rate drops considerably after 20 minutes, and that the concentration curve rises above the absolute excretion curve at this time, to drop below again after 50 minutes, when the urine rate has regained its original speed.

When we plot the milligrams of dye excreted during 20, 30, 60 and 90 minutes against the volumes of urine excreted during this time (in all our experiments), we find a definite increase in the dye excretion with increasing water diuresis (Text-fig. 3). This increase is most

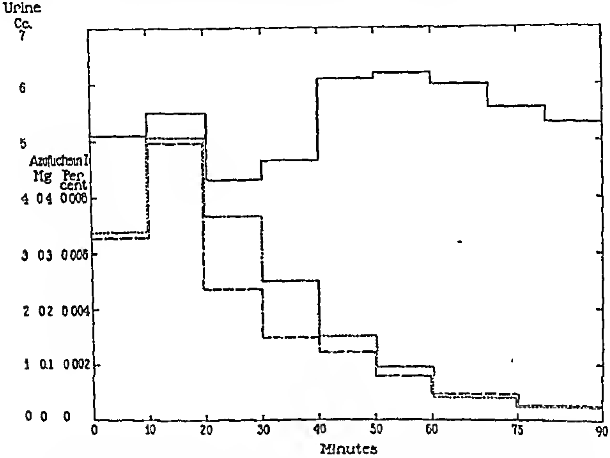


TEXT-FIG. 4. Concentration percentages of cyanol as compared with the urine volumes during the first 20 (*a*), 30 (*b*), 60 (*c*) and 90 (*d*) minutes of the experiments.

marked after 20 and 30 minutes and least so after 90 minutes. If we plot the concentration of the dye against the urine volume, we get similar results (Text-fig. 4). We then get curves which at low urine volume, are considerably below, and at high volume, above hyperbolas which have been constructed with the formula $x = \frac{k}{y}$, where x is the average concentration of the dye, and y the average urine volume, k being equal to the average mg. excreted times 100.[3]

[3] For the dead space in our experiments which we estimated as approximately 3 cc., the hyperbolas have been calculated only from urine volumes that amounted to at least 7 cc. Though it is obvious that there remains an error which becomes smaller with increasing urine volume, it can be seen from Text-figs. 4 and 7 that for any urine volume above 7 cc. this error is rather constant.

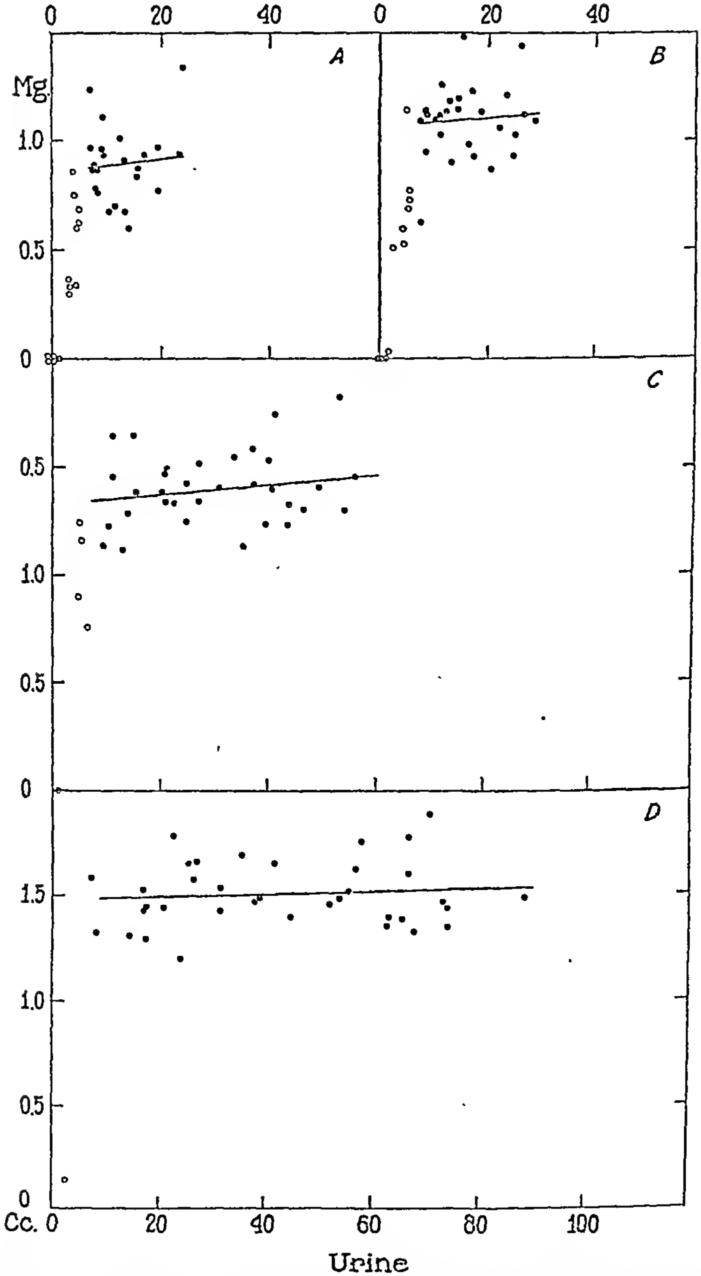
3. The excretion of *azofuchsin I* has been studied in 38 experiments (in 33 animals). In 10 experiments the peak of the excretion was observed in the first 10 minutes after the injection of the dye; in 21 experiments in the second 10 minute period. In all these experiments the urine rate amounted to at least 0.15 cc. per minute during the first 20 minutes. In the remaining 7 experiments in which the peak was reached later, the urine rate was 0.15 cc. or less. The larg-



TEXT-FIG. 5. Average quantities of urine (————) and azofuchsin I (mg. – – – – –, concentration per cent ....) excreted in 10 minute periods after the intravenous injection of 2 mg. of the dye.

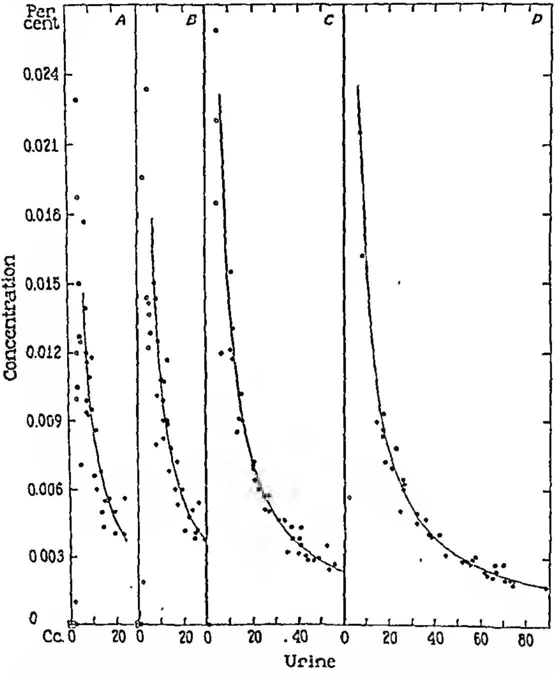
est amount of dye excreted during a 10 minute period was 1.05 mg., the highest concentration 0.04 per cent.

If, as in the case of cyanol, those experiments are taken into account in which the urine rate amounted to more than 0.15 cc. per minute, we find an average excretion curve as outlined in Text-fig. 5. With this dye also there is but a slight depression in the urine volume after 20 minutes. This, as in the case of cyanol, leads to a discrepancy between the concentration and absolute excretion curves.



TEXT-FIG. 6. Milligrams of azofuchsin I excreted during the first 20 (*a*), 30 (*b*), 60 (*c*) and 90 (*d*) minutes of the experiments as compared with the cc. of urine produced during these periods.

When we plot the mg. of dye excreted during 20, 30, 60 and 90 minutes against the volumes of urine excreted during this time, we find, unlike the case of cyanol, only a slight rise in dye excretion with



TEXT-FIG. 7. Concentration percentages of azofuchsin I as compared with the urine volumes during the first 20 (*a*), 30 (*b*), 60 (*c*) and 90 (*d*) minutes of the experiments.

urine volumes above 7 cc. (Text-fig. 6). When we plot the concentration of the dye against the urine volume, we have similar results; namely, curves which closely follow hyperbolas constructed as above (Text-fig. 7).

Variations in body weights, 86 per cent of which varied from 1600 to 1980 gm., appeared to exert no distinct differences, either in the cyanol or in the azofuchsin experiments.

DISCUSSION

From the foregoing results it is obvious that cyanol and azofuchsin I are excreted in a very different manner. Whereas the cyanol excretion amounted to no more than an average of 0.218 mg. in 20 minutes and 0.502 mg. in 90 minutes, *i.e.*, 10.9 per cent and 25.1 per cent of the injected dye, the corresponding figures for azofuchsin were 0.889 and 1.504 mg., *i.e.*, 44.4 per cent and 75.2 per cent. Whereas the cyanol excretion was slow (flat curve), azofuchsin was excreted very rapidly (steep curve; compare Text-figs. 2 and 5). Whereas the cyanol excretion depended on the urine volume, the azofuchsin excretion was essentially independent of this factor.

To evaluate these differences, we may compare our findings with available published data in the recent literature. If *cyanol* is a glomerular dye, its excretion should compare with a clearance which is regarded as a true measure of glomerular filtration, such as the inulin clearance, the curve of which in rabbits has been given by Kaplan and Smith (1935). As in our experiments there was a dead space which has been estimated as approximating 3 cc. of urine, we first corrected this error by using the formula m_2 (corrected mg.) $= \frac{v \cdot m}{v-3}$, v being the urine volume in cc., and m the actual amount of dye excreted in mg. The clearance (cc. of plasma cleared per minute) was then calculated by dividing the corrected mg. (m_2) by the number of minutes in which they were excreted, and by dividing this rate by the average mg. of dye contained in 1 cc. of plasma during this period, *i.e.*, by $\left(2.0 \text{ (mg. of dye injected)} - \frac{m_2 \text{ (mg. of dye excreted)}}{2}\right) : 100$ (100 being the approximate number of cc. of plasma contained in our rabbits). A further correction was necessary because we frequently found a considerable amount of dye in the small intestines, when the animals were killed 2 hours after the injection of the dye; we believe that this fraction, which was probably excreted by the liver,

may amount up to 50 per cent of the total dye; therefore, another clearance (2) was calculated by dividing the rate by $\left(2.0 - \frac{2m_2}{2}\right)$: 100. Since the data of Kaplan and Smith were given on the basis of square meters of body surface, and since their animals were larger than ours, we finally divided our clearances by the average body surface of our animals which according to the table of Taylor, Drury and Addis (1923) amounted to about 0.1415 sq. m.

If now we compare our clearances thus derived from various points of Text-fig. 3 *a* and *b* with the inulin clearances of Kaplan and Smith, we find, considering the values after 20 minutes only (Text-fig. 3 *a*), that at urine rates of 2.47 to 9.89 cc. per minute per sq.m. the cyanol clearances amount to an average of from 8.1 to 8.9 per cent of the inulin clearances (Table III, 3 *a*), and if we consider the values after 30 minutes only (Text-fig. 3 *b*), that at urine rates from 3.06 to 8.25 cc. they amount to an average of from 6.8 to 7.5 per cent (Table III, 3 *b*). In both instances the deviations from the average are remarkably small. However, if we consider the values at urine rates below 2.47 cc. per minute per sq. m., we find considerably higher cyanol clearances as compared with the corresponding inulin clearances (Table III, 3 *a*,*b*,*c*). The explanation for this may be found in the "dead space" of our experiments, for it is obvious that the dye which is excreted diffuses in the urine contained in the dead space, and that at a very slow urine flow the diffusion introduces an error which considerably elevates the corrected excretion rate.[4] It may be noted that this error is of importance when the urine rate is below 1.65 to 2.47 cc. per minute per sq. m., which corresponds closely to the point below which urine volumes have been omitted in the calculation of the hyperbolas in Text-figs. 4 and 7.[3]

Comparison of our clearances with the xylose clearances of Kaplan and Smith shows no correlation (Table III). We find, for example, that after 20 minutes (Table III, 3 *a*) with an increase in diuresis from 2.47 to 9.89 cc. per minute per sq. m., the cyanol clearance in-

[4] That this actually occurs is illustrated by the observation of dye in as little as 1.2 cc. of urine 10 minutes after the injection of the dye, the catheter holding 1.5 to 2.0 cc. of urine.

creases 24 to 30 per cent, and the inulin clearance 26 per cent,[5] whereas the xylose clearance increases as much as 74.6 per cent. From all these figures it is evident that the cyanol excretion, as far as its curve is concerned, closely resembles the inulin clearance, whereas the xylose clearance follows a very different curve. We regard this as evidence that cyanol is handled by the kidney of the rabbit as are inulin and

TABLE III

Cyanol Clearance as Compared with Corresponding Inulin and Xylose Clearances as Obtained by Kaplan and Smith

Fig. from which data were derived	Urine	Dye	Period	Urine per min. per sq. m.	Dye excretion (corrected): Amount	Clearance: Per min. 1	Per min. 2	Per. min. per sq. m. 1	Per. min. per sq. m. 2	Amount as compared with inulin clearance 1	Amount as compared with inulin clearance 2	Amount as compared with xylose clearance 1	Amount as compared with xylose clearance 2
	cc.	*mg.*	*min.*	*cc.*	*mg.*	*cc.*	*cc.*	*cc.*	*cc.*	*per cent*	*per cent*	*per cent*	*per cent*
3 *a*	4	0.075	20	1.41	0.300	0.818	0.882	5.8	6.2	11.2	11.9	24.2	25.8
"	7	0.150	20	2.47	0.262	0.701	0.754	5.0	5.3	8.4	8.9	14.9	15.8
"	12	0.200	20	4.24	0.267	0.717	0.770	5.1	5.7	7.7	8.6	11.6	13.0
"	20	0.260	20	7.07	0.306	0.828	0.903	5.9	6.4	8.1	8.8	11.0	12.0
"	28	0.290	20	9.89	0.325	0.884	0.970	6.2	6.9	8.3	9.2	10.6	11.8
3 *b*	4	0.100	30	0.92	0.400	0.739	0.831	5.2	5.9	11.1	12.6	28.1	31.9
"	7	0.180	30	1.65	0.315	0.570	0.623	4.0	4.4	7.4	8.1	15.4	16.9
"	13	0.250	30	3.06	0.325	0.589	0.647	4.2	4.6	6.7	7.4	11.2	12.3
"	20	0.300	30	4.71	0.353	0.645	0.715	4.6	5.1	6.8	7.5	10.0	11.1
"	35	0.350	30	8.25	0.383	0.706	0.790	5.0	5.6	6.8	7.6	8.9	10.0
3 *d*	7	0.450	90	0.55	0.787	0.544	0.721	3.8	5.1	9.3	12.4	27.1	36.4
3 *c*	7	0.275	60	0.83	0.481	0.456	0.228	3.2	3.7	7.0	8.0	18.3	21.1

creatinine, the clearances of which appear to be true measures of filtration (Kaplan and Smith, 1935). If this is correct, then we may estimate that in rabbits at plasma levels of about 2 mg. per cent no more than 10 per cent of the dye is available for filtration.

If we turn now to *azofuchsin I*, we see at once that there is no correlation between its rate of excretion and that of inulin, creatinine

[5] These figures have been calculated from the data of Kaplan and Smith.

or xylose. It is true that the concentration of this dye also rises with increasing urine volume (Text-fig. 6), but this rise does not exceed that which is caused by the dead space in our experiments. If we calculate the clearance as above, we find that 20 minutes after the injection of the dye the clearance amounts to about 21 to 24 cc. per minute per sq. m., *i.e.*, about 34 to 39 per cent of the inulin clearance at a urine flow of 2.47 cc. per minute per sq. m., or 28 to 32 per cent at a flow of 8.48 cc.; after 30 minutes it amounts to 18 to 20 cc. per minute per sq. m., *i.e.*, 33 to 37 per cent of the inulin clearance at a urine flow of 1.65 cc. per minute per sq. m. or 25 to 28 per cent at a flow of 7.07 cc. It is self evident that the differences in these figures at different urine flows cannot be explained by reabsorption. In this case the reverse should be expected, namely a smaller clearance with low urine flow and *vice versa*. It appears to be evident, therefore, that the excretion of azofuchsin I cannot be explained by filtration alone.

However, if we compare the excretion of azofuchsin with that of phenol red which is known today to be mainly secreted, we find a close correlation in at least three respects.

1. Whereas in man after intravenous injection Rowntree and Geraghty (1909–10, 1912) recovered 68 per cent of the injected phenol red in the urine 1 hour after the injection; Shaw (1925) 40 per cent after 15 minutes, 57 per cent after 30 minutes, 69.5 per cent after 1 hour and 74 per cent after $1\frac{1}{2}$ hours; and Chapman and Halsted (1933) 36 per cent after 15 minutes, 54 per cent after 30 minutes, and 66 per cent after 1 hour; we recovered 44 per cent of the injected azofuchsin after 20 minutes, 54 per cent after 30 minutes, 70 per cent after 1 hour, and 75 per cent after $1\frac{1}{2}$ hours. Furthermore, as after subcutaneous or intramuscular injection the amount of phenol red excreted in 1 hour was 50 to 51 per cent in man, dogs and rabbits (Rowntree and Geraghty, 1909–10, 1912; Eisenbrey, 1911; Frothingham, Fitz, Folin and Denis, 1913), it appears that the values in man and in rabbits are comparable, which would mean that phenol red and azofuchsin I both have the same excretion curve.

2. Like the azofuchsin excretion the elimination of phenol red is essentially independent of the urine volume. For phenol red this has been demonstrated in toadfish, frogs, rabbits, dogs and man

(Rowntree and Geraghty, 1909–10; Marshall and Kolls, 1919; Cushny, 1926; Scheminzky, 1929; Marshall and Grafflin, 1932; Chapman and Halsted, 1933; Shannon, 1935).[6]

3. If we assume that azofuchsin like phenol red (Chambers and Kempton, 1933; Richards, 1935) and cyanol (see below) is not reabsorbed, and that, as in the case of phenol red (Grollman, 1925, 1925–26), in rabbits at plasma concentrations below 2 mg. per cent, 95 per cent of the dye is bound by the plasma proteins, we arrive at azofuchsin clearances which are considerably higher than those mentioned above. 20 minutes after the injection of the dye they would amount to 680 to 780 per cent of the inulin clearance, at a urine flow of 2.47 cc. per minute per sq. m., or to 560 to 640 per cent, at a flow of 8.48 cc. If the inulin clearance represents the filtration rate, this would mean that at the lower urine flow 85 to 90 per cent of the excreted dye was secreted, and at rapid flow 82 to 84 per cent. If we compare these figures with the phenol red figures available in the literature we find a close correlation. In dogs, Shannon (1935) found the secreted proportion to approximate 83 per cent, at plasma levels of 0.5 to 1.5 mg. per cent. Similar figures can be calculated from Marshall's data (1932), namely 82 to 90 per cent, at plasma levels of 0.21 to 0.54 mg. per cent, or from Sheehan's data (1936), namely 70 to 90 per cent, at plasma levels of 0.37 to 3.00 mg. per cent, both in dogs. In man, Goldring, Clarke and Smith (1936) found the secreted proportion to amount to 95 per cent, at plasma levels below 1 mg. per cent. For the rabbit, comparable data appear to be lacking, excepting the studies of Elsom, Bott and Walker (1937) who investigated the renal blood flow and excretion of phenol red; however, it is difficult to compare these results with ours because of very different experimental conditions employed.

We believe that these figures present evidence that azofuchsin is mainly secreted. If this is true, the drop in the calculated secretion

[6] If MacKay and Oliver (1930) found the phenol red excretion in rabbits dependent on the urine volume, this is explained by the tremendous amount of dye they injected. Since it has been generally found that the secretory capacity of the tubules is limited (in dogs apparently at 0.4 mg. per 100 cc. of plasma (Sheehan, 1936)), it can be calculated from Sheehan's figures that at a plasma concentration of 40 mg. per cent the filtration exceeds the secretion 10 times.

ratio from 85 to 90 per cent at slow urine flow to 82 to 84 per cent at rapid flow would indicate either that the diffusibility of azofuchsin is higher than that of cyanol, or, if the drop actually occurs, that in rabbits the secretion also depends on the rate of urine formation. The latter possibility could be easily understood, for at high urine flow there is a considerable dilatation of the tubules which cannot be without influence on the function of the epithelial cells.

In attempting to answer the question whether our deductions are mere hypotheses, or whether they are supported by convincing or conclusive evidence, we have to consider that the kidney has three major functions; namely, filtration, secretion and reabsorption. If the assumption is correct that the inulin clearance is a true measure of glomerular filtration, if the inulin clearance curve of Kaplan and Smith represents the actual filtration curve at different urine rates, and if our determinations are accurate enough and sufficient in number, it appears that in the case of cyanol, reabsorption can be ruled out, for it would depress the clearance values at low urine flow. In our experiments, however, the reverse was observed, that is an actual increase when the urine flow sank below 2.47 cc. per minute per sq. m. As to a possible secretion of cyanol, the evidence is not so striking. But if we consider the literature (page 750) and if we recall that with an increase in the urine rate from 2.47 to 9.89 cc. per minute per sq. m. the clearance curve of the dye closely followed the curve of the inulin clearance, whereas the clearance of azofuchsin varied from 34 to 39 per cent to less than 28 to 32 per cent at the same rates, we feel that the evidence that cyanol is only or mainly filtered is at least strongly suggestive.

Concerning azofuchsin I, it has been shown that its excretion is fundamentally different from that of cyanol, inulin or xylose. Conclusive evidence has been presented that this difference cannot be explained by reabsorption of the dye (page 763). Logically there remains, then, only the possibility that azofuchsin is secreted in part, a conclusion which is corroborated by the close resemblance of its excretion to that of phenol red. Concerning the relative amount secreted, no definite information is available at present. However, from the data presented it appears that this percentage is not much different from that of phenol red.

SUMMARY

1. The excretion of water has been studied in a large number of experiments on rabbits. After the ingestion of 100 cc. of water, the day before the experiment and as part of the experiment, the average diuresis amounted to 0.6 cc. per minute during a half hour period. The highest individual rate was 1.5 cc. per minute.

2. The excretion of cyanol and of azofuchsin I has also been studied. It has been shown that the cyanol excretion curve closely parallels the inulin excretion curve of Kaplan and Smith. Evidence is presented that cyanol is disposed of entirely or mainly by filtration.

3. The excretion of azofuchsin I is not only very different from that of cyanol or inulin, but almost identical with that of phenol red. Evidence is presented that at low plasma concentration azofuchsin is, in the main, secreted.

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ACUTE EXPERIMENTAL GLOMERULAR NEPHRITIS IN RABBITS: A CORRELATION OF MORPHOLOGICAL AND FUNCTIONAL CHANGES*

By WILLIAM E. EHRICH, M.D., RICHARD E. WOLF, and GEORGE M. BARTOL

(*From the Department of Pathology, University of Pennsylvania, School of Medicine, Philadelphia*)

PLATES 32 TO 35

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Of the two methods of correlating morphologic and functional changes in renal disease, clinical and experimental, the former has been repeatedly utilized with but moderate success, for when most patients die it is difficult to interpret the complex morphological alterations in the kidneys. The application of the experimental method, on the other hand, has been limited by our inability to reproduce the more important disorders. Necrotizing nephrosis, to be sure, has frequently been produced; but in this condition the lesions are so different from those of common renal disease that comparable conclusions are of little value. Only since the recent discovery of Masugi (1933) that glomerular nephritis can be produced by the injection of anti-kidney serum, could this avenue of approach be regarded as open. It may thus be attempted once more to correlate certain morphologic and functional changes.

In acute glomerular nephritis in man, as is well known, the tubules may be little if at all altered morphologically. It should be possible now to ascertain by experiment whether or not they are impaired functionally, or, in other words, whether glomeruli and tubules can function independently of one another. Another item which appears ready for investigation is the much disputed question as to whether the initial impairment of glomerular function in glomerular nephritis

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is due to spasms of the arterioles, as maintained by Volhard (1931), or to the anatomical changes of the loops. This question has been attacked already by Fahr (1934, 1935) and his pupils Hemprich (1935) and Weiss (1935–36), as well as by Tsuji (1936–37); but as their evidence is only morphologic, this question cannot be looked upon as settled. Finally, attempts may now be made to ascertain whether common clinical findings such as edema, albuminuria and hematuria can be correlated with histological changes.

Method

The following method (Ehrich, 1937) was used to produce glomerular nephritis: Peking ducks were immunized with 16 to 20 doses of 5 to 15 cc. of an emulsion which was prepared by suspending the blood free mash of the kidneys of a rabbit in 60 cc. of normal saline solution. 4 to 8 days after the last injection the ducks were decapitated, the blood collected and the serum separated and inactivated by heating to 56°C. for 30 minutes; 0.5 to 7.0 cc. were then injected intravenously into rabbits either once or repeatedly at intervals of 2 days. Most rabbits were Chinchillas weighing 1500 to 2000 gm.

Though there was a considerable variation in the response of different animals, it can be seen from Table I that the effect of the sera depended partly on the dosage with which the ducks were immunized. Serum 9, for example, was so toxic that a single dose of 1 cc. resulted in the death of one rabbit, whereas 7 cc. of sera 1 and 5 failed to kill. With repeated doses larger amounts could be given; but then a number of animals died in what appeared to be acute anaphylactic shock. This has been observed as early as 7 days after the first injection, in spite of the fact that the rabbits were injected every 2nd day.

The cause of the toxicity has not been detected. As the animals developed an acute anemia, the possibility cannot be excluded that the toxicity was due to substances other than the nephrotoxin. However, it might just as well be a matter of the strength of the serum, for Smadel (1936, 1937) found in rats a severe anaphylactoid reaction either as a result of giving a comparatively large amount of a relatively pure nephrotoxic serum or a smaller amount of serum rich in non-organ-specific anti-rat-tissue antibodies as well as in the more specific nephrotoxin.

The function of the kidneys was tested by water and dye tests, described in the preceding paper. Cyanol was used as a measure of filtration, azofuchsin I as a measure of secretion. 100 cc. of water were given by stomach tube, and the urine collected every 15 or 30 minutes. Usually, 1 hour after the ingestion of water, 2 cc. of a 0.1 per cent solution of cyanol or azofuchsin were injected intravenously, and thereafter the urine collected every 10 minutes. In addition, we determined the reaction of the urine, the presence of protein and in some animals the blood urea nitrogen.

In the microscopic sections special attention was paid to the size of the glomeruli

TABLE I

The Effect of Different Sera on Different Rabbits

Serum No.	Doses of kidney juice injected into ducks		Doses of serum injected into rabbits
	cc.	*times*	
1	5	16	3.5/1, 7.0/1
2	5	20	3.5/1, (7.0/1)
3	10	16	3.5/1, (7.0/1)
4	10	18	5.0/1
5	10	20	3.5/1, 7.0/1
6	15	15	2.5/1, 3.5/1, 4.0/1, 7.5/3, (7.5/3), (7.8/3)*
7	15	18	(5.0/1)
8	15	18	5.0/1
9	15	20	(1.0/1), (2.0/1), (3.0/1), (3.5/4)*, (3.5/4)*, (3.5/4)*, (3.5/4)*, 4.0/5, (4.0/5), 6.5/6
10	Mixture of sera 4, 7 and 8		2.5/1, 4.0/1, (4.0/1), 5.0/1, 5.0/1, 5.0/1, (5.0/1), (6.0/1), 7.0/2, 9.0/3, 11.0/3 (3.0/1), (4.0/1), 5.0/1, 5.0/1, 5.0/1, (5.0/1), (5.0/1), 7.0/2, 9.0/3, 9.0/3

( ) = died within 48 hours after the last injection.
Figure at left of the oblique line = total cc. of serum injected; figure to right of the line = number of injections given.
* Died in acute anaphylactic shock.

TABLE II

The Excretion of Water, Cyanol and Azofuchsin I during Different Periods of the Disease

Period	Animal No.	Serum injected		Duration of disease	Excretion of water		Excretion of cyanol								Excretion of azofuchsin I							
					Water given the day before	Amount excreted in 24 hrs.	Amount excreted in				Amount of urine in the corresponding				Amount excreted in				Amount of urine in the corresponding			
							20 min.	30 min.	60 min.	90 min.	20 min.	30 min.	60 min.	90 min.	20 min.	30 min.	60 min.	90 min.	20 min.	30 min.	60 min.	90 min.
		No.	*cc.*	*days*		*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
A	1-01	10	5	1	0	2.5	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	1-02	10	5	1	0	3.0	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
B	1-07	10	5	3	0	81.5	—	—	—	—	—	—	—	—	0.984	1.240	1.440	1.537	14.4	20.8	24.0	39.4
	1-04	10	5	3	0	74.3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	1-12	10	9	6	0	20.3	—	—	—	—	—	—	—	—	0	0	1.000	1.600	0.1	0.9	3.3	19.1
	7-0	8	5	7	0	75.8	0.292	0.380	0.498	0.563	18.0	26.8	40.0	50.4	—	—	—	—	—	—	—	—
	1-11	4	5	7	0	118.2	—	—	—	—	—	—	—	—	0.480	0.819	1.308	1.502	15.7	27.0	51.0	76.2
C	1-12	10	9	7	+	6.4	0	0	0.180	0.225	1.2	1.7	4.1	4.9	—	—	—	—	—	—	—	—
	1-21	10	7	8	0	7.8	0	0	0.111	0.329	1.2	1.7	4.1	8.0	—	—	—	—	—	—	—	—
	1-21	10	7	9	+	4.2	—	—	—	—	—	—	—	—	0	0.016	0.216	1.085	1.4	1.6	2.2	3.5
	1-20	10	7	9	+	16.2	0	0.020	0.244	0.289	1.4	2.4	7.1	12.6	—	—	—	—	—	—	—	—
	1-12	10	9	10	0	39.0	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	1-20	10	7	10	+	37.7	—	—	—	—	—	—	—	—	0.950	1.070	1.230	1.348	11.1	14.1	20.1	31.5
	9-0	10	11	11	0	30.6	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	1-00	10	9	11	0	15.2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	1-12	10	9	11	+	19.8	0.108	0.133	0.244	0.326	4.4	4.9	7.0	9.4	—	—	—	—	—	—	—	—
	1-20	10	7	11	+	33.3	0.175	0.243	0.316	0.340	4.2	7.6	17.6	29.7	—	—	—	—	—	—	—	—
	1-99	9	4	11	+	47.1	—	—	—	—	—	—	—	—	0.485	0.941	1.332	1.470	3.7	7.5	17.3	40.3

D	1-88	6	4	12	+	62.0	—	—	—	—	—	—	—	—	0.492	0.992	1.517	1.657	4.9	9.9	32.7	48.7
	1-99	9	4	12	+	62.2	0.125	0.214	0.384	0.477	6.9	10.6	23.1	38.6	—	—	—	—	—	—	—	—
	1-88	6	4	13	+	66.6	0.179	0.226	0.442	0.513	14.6	17.2	28.8	50.8	—	—	—	—	—	—	—	—
	1-74	6	7.5	14	+	77.8	0.184	0.226	0.282	0.311	16.6	23.6	36.4	47.0	—	—	—	—	—	—	—	—
	1-00	10	9	15	0	93.8	0	0.051	0.230	0.300	1.4	8.2	36.4	62.6	—	—	—	—	—	—	—	—
	1-86	6	5.5	20	+	47.3	0.032	0.084	0.299	0.396	2.6	5.2	22.2	36.1	—	—	—	—	—	—	—	—
	2-02	9	6.5	20	+	70.2	—	—	—	—	—	—	—	—	0.723	1.083	1.425	1.582	9.6	16.8	33.0	50.5
	1-86	6	5.5	21	+	56.8	—	—	—	—	—	—	—	—	0.758	1.108	1.518	1.661	8.2	13.2	27.6	44.6
	2-02	9	6.5	21	+	72.8	0.172	0.218	0.320	0.370	15.3	19.1	32.5	49.0	—	—	—	—	—	—	—	—
E	7-1	10	5	23	0	1.5	0	0	0	0.045	0.4	0.4	0.9	2.0	—	—	—	—	—	—	—	—
	1-00	10	9	23	0	6.8	—	—	—	—	—	—	—	—	0	0.036	0.436	0.709	0	1.2	4.3	9.9
	7-1	10	5	24	+	23.1	—	—	—	—	—	—	—	—	0	0.660	1.577	1.758	0.5	2.7	14.4	33.4
	1-00	10	9	24	+	16.5	0.120	0.210	0.338	0.367	3.5	5.5	10.6	17.4	—	—	—	—	—	—	—	—
	7-1	10	5	25	+	5.1	0	0	0.050	0.325	0.4	1.0	2.2	5.4	—	—	—	—	—	—	—	—
	1-00	10	9	25	+	15.8	—	—	—	—	—	—	—	—	0	0.135	0.570	1.121	2.0	3.8	6.7	14.4
F	1-74	6	7.5	28	+	32.2	0.048	0.110	0.265	0.332	3.2	5.8	19.3	28.4	—	—	—	—	—	—	—	—
	1-74	6	7.5	29	+	32.5	—	—	—	—	—	—	—	—	0.766	0.991	1.394	1.602	5.7	8.2	13.4	25.0
	1-02	10	5	31	0	50.5	—	—	—	—	—	—	—	—	0.600	0.990	1.384	1.525	3.3	5.9	15.9	27.5
	1-04	10	5	31	0	41.7	0.076	0.195	0.342	0.397	2.1	3.5	10.7	24.2	—	—	—	—	—	—	—	—
	1-02	10	5	32	+	12.7	0.126	0.252	0.312	0.355	3.1	5.9	8.0	15.8	—	—	—	—	—	—	—	—
	1-04	10	5	32	+	64.4	—	—	—	—	—	—	—	—	0.954	1.161	1.501	1.819	14.6	19.2	23.8	42.3
	1-74	6	7.5	43	+	99.9	—	—	—	—	—	—	—	—	0.990	1.140	1.486	1.635	17.9	22.9	42.9	71.4
	1-74	6	7.5	44	+	99.9	0.204	0.270	0.391	0.428	13.7	22.8	52.8	68.8	—	—	—	—	—	—	—	—
Normal average……					+	59.7	0.218	0.267	0.410	0.502	—	—	—	—	0.889	1.089	1.393	1.504	—	—	—	—

and to the number of their nuclei. Both were determined by the method of Ehrich and Sommer (1933) by measuring the two largest diameters, which were perpendicular to each other, of 10 to 20 tufts the afferent or efferent vessels of which were met in the slides, and by counting the number of nuclei in the same tufts. Because the size of the central glomeruli is slightly larger than that of the peripheral ones (Peter, 1909), we proceeded as in the case of differential counts of leucocytes by moving the slides from capsule to medulla and *vice versa*. It should be noted that all glomeruli with fibrin or fibrosis were omitted in these counts.

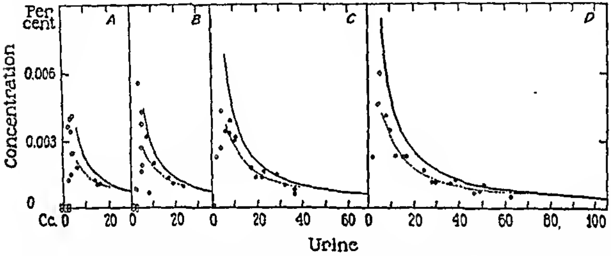
RESULTS

1. The *excretion of water* has been tested in 41 experiments on 17 rabbits, from 1 to 44 days after induction of the disease. During the first week of the experiment, with the exception of the first day,[1] the diuresis was essentially normal, but thereafter it was markedly depressed (Table II). After the 11th to 25th day the diuresis was again found to be normal. It is true that during any one period the individual figures were quite as variable as in normal animals (Ehrich, Bartol and Wolf, 1938). However, if we compare different periods, we find that the differences are not caused by the normal variations alone, but by a shift in the whole range of variations. It should be noted that it made no difference whether or not the animals had been given water the day before the experiment, but that all animals behaved as if they were "wet" animals. The explanation for this may be found in the fact that they already had edema or were in a state of *Oedembereitschaft* (*i.e.*, ready to develop edema) (see page 777).

2. The *excretion of cyanol* has been studied in 19 experiments, in 13 rabbits, from 7 to 44 days after the injection of serum. With the exception of the first and of the last experiment, practically all figures are considerably lower than normal (Table II). Since the amount of cyanol excreted during a certain period depends in part on the urine volume (previous article), the two have been compared by plotting the concentration of the dye against the cc. of urine excreted in the corresponding periods (Text-fig. 1). It is seen that almost all the figures are either in the lowest region of the normal range or below it; or, in other words, that the diminution in cyanol excretion is greater than could be explained by the diminution in urine formation alone.

[1] Three additional rabbits tested on the 1st or 2nd day excreted no urine whatsoever during the experiment.

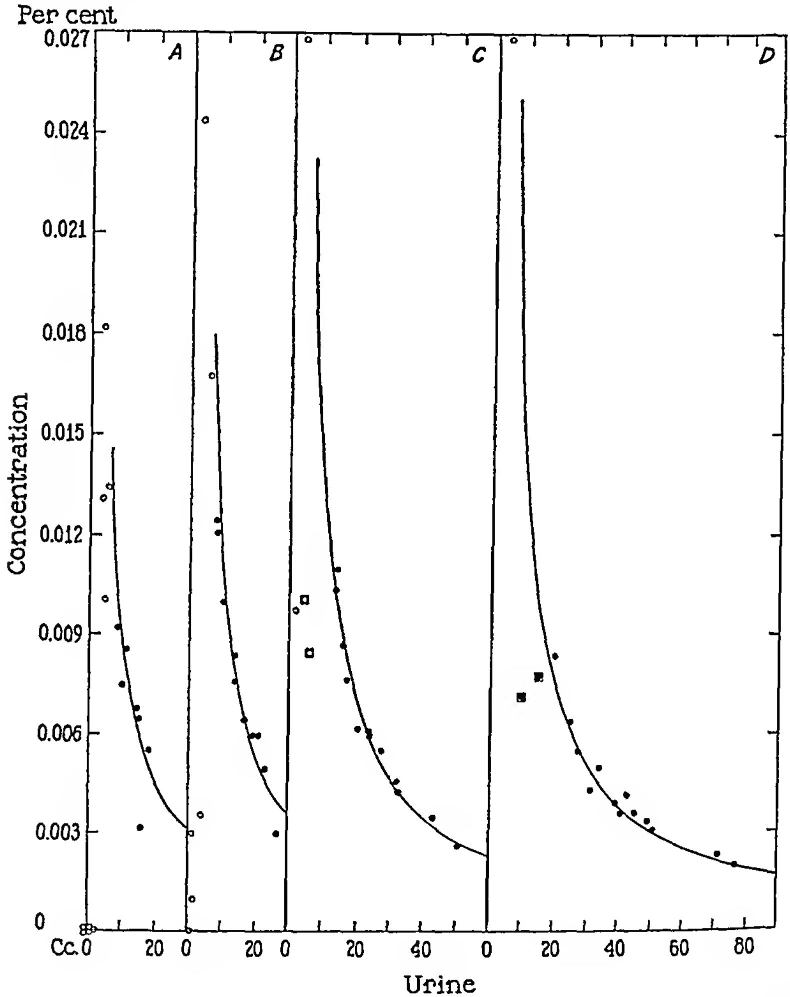
3. The *excretion of azofuchsin I* has been studied in 16 experiments, in 14 rabbits, from the 3rd to the 43rd day of the disease. It has been found that in almost all instances it was normal at all times. There was no change in the amount excreted (Table II), nor in the concentration as compared with the urine volume (Text-fig. 2). There was but one exception, namely rabbit 1-00, which showed a decreased azofuchsin excretion on the two occasions at which this was measured. It will be seen later that this animal was the only one which showed a marked fatty degeneration of the tubules (see page 782).



TEXT-FIG. 1. The excretion of cyanol from the 7th to the 32nd day of the disease. The concentration percentages of the dye during the first 20 (*a*), 30 (*b*), 60 (*c*) and 90 (*d*) minutes of the dye experiments as compared with the cc. of urine produced during these periods. ——— hyperbola calculated from the average amount of dye excreted normally; -------- lowest limit of normal range.

4. The *presence of protein* in the urine, as determined by boiling, was studied in 46 experiments in 21 rabbits. Whereas in the first week after the injection of the serum there was but a trace of protein in most experiments, in the 2nd week there was a marked proteinuria in most instances (Table III). After the 3rd week there was either no protein or but a trace, with the exception of rabbit 1-00 and perhaps rabbit 7-1, which are the same rabbits which showed a decrease in cyanol excretion even at this time. If we divide the experiments into periods, as in Table II, and if we compare the two tables, we find that both proteinuria and water and cyanol retention started at the same time; namely, 1 week after the injection of the serum.

5. The *reaction of the urine*, as determined by litmus paper, was studied in 20 experiments, in 12 animals. Although with a very small



TEXT-FIG. 2. The excretion of azofuchsin I in acute glomerular nephritis (all experiments). The concentration percentages of the dye during the first 20 (*a*), 30 (*b*), 60 (*c*) and 90 (*d*) minutes of the dye experiments as compared with the cc. of urine produced during those periods. ———— hyperbola calculated from the average amount of dye excreted normally; □ data from rabbit 1-00.

diuresis an acid reaction was found, and though with an acid reaction the diuresis tended to be smaller than with an alkaline reaction, this

was by no means constant. There was no distinct relation to the phases of the disease. However, in the cases which were protein-free the urine gave an alkaline reaction in most instances, whereas with proteinuria it invariably gave an acid reaction in all cases tested (Table III).

6. The *blood urea nitrogen* concentration in the blood was tested in 21 experiments, in 10 animals. In most instances the figures were well within the normal range (9 to 20 mg. per cent). However, of four tests made between the 6th and 10th day of the disease, two were slightly increased and two were as high as 60 and 105 mg. per cent.

7. As to the *gross changes* in the tissues of our animals, it should be noted that on the first day after the injection of the serum we frequently observed a marked edema of penis and scrotum; and on dissection, an increased amount of fluid in the abdominal and thoracic cavities. In the 2nd week and thereafter we frequently found ascites, hydrothorax and hydropericardium. The kidneys were not much changed during the first few days. However, in the animals killed on the 6th day and thereafter they were swollen, pale or yellowish brown in color, and showed fine blood dots on their surface. After the 4th week the blood dots were less conspicuous; and after the 6th week they were absent.

8. For the *histology* the kidneys of 30 rabbits were studied. The size of the glomeruli and the number of their nuclei increased during the first week to be more than doubled after 8 days (Figs. 1 and 2), and later they decreased again (Table IV). The number of granulocytes contained in the glomeruli varied a great deal; in the first 4 weeks of the disease, however, they were distinctly elevated in at least 70 per cent of the cases. Fibrin was present in the glomeruli (Fig. 2 *d*) from 8 to 12 days after the injection of the serum, in 7 of 8 animals; the number of glomeruli affected varied from 1 per cent to 53 per cent. Capsular adhesions, crescents or fibroses (Figs. 3 and 4) were observed from the 8th day onward; they were present in 15 of 23 animals, in 3 per cent to 92 per cent of the glomeruli. The amount of blood contained in the glomeruli varied greatly, partly because some animals died spontaneously, whereas the others were killed by a blow on the neck. But apart from this it can be seen that in the

TABLE III

The Excretion of Protein and the Reaction of the Urine in Different Periods after the Injection of the Serum

Period	Animal No.	Serum injected		Duration of disease	Protein in urine	Reaction of urine
		No.	*cc.*	*days*		
A	1-01	10	5	1	Trace	Acid
	1-02	10	5	1	"	"
	1-12	10	9	6	0	–
	9-4	3	3.5	6	+++	–
	7-0	8	5	7	Trace	Acid
	1-11	4	5	7	"	"
	9-3	1	7	7	+	–
B	1-21	10	7	8	+++	–
	1-20	10	7	8	+++	–
	1-20	10	7	9	+	–
	9-3	1	7	9	+	–
	9-4	3	3.5	9	+++	–
	1-12	10	9	10	+++	–
	9-0	10	11	11	+++	–
	1-00	10	9	11	+++	–
	1-12	10	9	11	++	–
	1-20	10	7	11	+++	–
C	1-99	9	4	11	0	Alkaline
	1-11	4	5	12	+++	–
	9-7	10	4	12	+	–
	7-0	8	5	12	++	–
	1-88	6	4	12	Trace	Acid
	1-99	9	4	12	0	"
	1-88	6	4	13	Trace	"
	1-00	10	9	15	++	"
	9-0	10	11	15	++	–
	1-86	6	5.5	20	0	Alkaline
	2-02	9	6.5	20	0	"
	1-86	6	5.5	21	+	Acid
	2-02	9	6.5	21	0	"
D	7-1	10	5	23	+	–
	1-00	10	9	23	++	–
	1-00	10	9	24	+++	–
	1-00	10	9	25	++	–

TABLE III—*Concluded*

Period	Animal No.	Serum injected		Duration of disease	Protein in urine	Reaction of urine
		No.	*cc.*	*days*		
E	1-74	6	7.5	28	+	Acid
	1-74	6	7.5	29	Trace	"
	1-02	10	5	31	0	–
	1-04	10	5	31	Trace	–
	1-74	6	7.5	43	0	Alkaline
	1-74	6	7.5	44	0	"
	3-3	5	7	47	+	–
	3-6	2	3.5	47	+	–
	9-4	3	3.5	61	0	–
	9-3	1	7	61	0	–
	9-4	3	3.5	63	+	–
	9-3	1	7	93	0	–

first week the glomeruli were hyperemic in most cases, that they then became anemic, and that after the 4th week they were well filled again in most instances (Table IV).

Tubular changes were chiefly evident in the lumina (Table IV). There were hyaline casts (Fig. 5) from the 2nd to the 7th week in 13 of 18 cases, and erythrocytes or blood casts in the tubules (or in the glomerular spaces or in both) (Fig. 6) in 12 of 18 cases. In the first week erythrocytes were seen in one instance only. After the 7th week they were absent with but one exception. As a result of the casts the tubules were frequently dilated (Fig. 7). Changes in the epithelial cells were observed in but 7 animals. In all these instances there were fatty changes (Fig. 8), and in two instances hyaline droplets as well (Fig. 9).

If we compare Table IV with Tables II and III, we find that the onset of oliguria, diminished cyanol excretion and marked proteinuria closely conforms to the period when proliferation and anemia within the tufts were at their peak. If we compare the tubular changes with those of the glomeruli, we see that the fatty changes closely parallel the deposition of fibrin or the adhesions, crescents or fibroses, whereas the hyaline casts or the erythrocytes or blood casts show but a partial correlation. However, if we compare the hyaline casts with the clinical findings, we find that they closely follow the proteinuria.

TABLE IV

Glomerular and Tubular Changes in Different Periods of the Disease

Animal No.	Weight		Serum injected		Duration of disease	Glomerular changes						Tubular changes			
	Beginning of experiment	End of experiment				Size	No. of nuclei	No. of granulocytes	Glomeruli having		Content of blood*	Hyaline casts	Erythrocytes, blood casts	Fatty change	Hyaline droplets
									Fibrin	Adhesion crescents					
	gm.	*gm.*	*No.*	*cc.*	*days*	μ^2			*per cent*	*per cent*					
1-01†	1840	1870	10	5	1	6940	92.5	10.2	0	0	+++	0	0	0	0
1-87	1800	1800	6	3.5	3	5502	89.3	0.8	0	0	++	0	0	0	0
1-15	1570	1100	10	9	6	6267	91.2	3.1	0	0	+	0	Medium	0	0
1-95†	1600	1620	9	3.5	7	7208	98.1	4.3	0	0	+++	0	0	—	0
1-96†	1880	1880	9	3.5	7	7337	111.5	3.7	0	0	+++	0	0	—	0
1-97†	1720	1610	9	3.5	7	6138	88.3	1.6	0	0	+++	0	0	—	0
1-19	2210	2160	10	7	8	13,478	180.5	0.9	53	30	+	Medium	Few	Slight	0
1-70†	1460	1520	6	8	8	9677	164.2	0.8	1	0	+	0	0	0	0
1-98†	1820	1740	9	3.5	9	7089	93.5	—	0	0	++	Few	0	0	0
1-21	1910	1690	10	7	10	11,310	148.4	3.8	38	28	+	"	Few	Slight	0
1-94†	1750	1660	9	4	11	6735	103.1	—	1	0	++	0	0	—	0
7-0	2180	2180	8	5	12	9161	129.3	3.1	14	35	+	Many	Medium	Moderate	Moderate
9-7	2200	2200	10	4	12	10,387	147.5	3.6	23	40	+	"	Few	"	"
1-11	1940	2150	4	5	12	8866	141.3	2.6	7	20	+	Few	0	Slight	0
1-88†	1710	1560	6	4	19	7014	106.2	4.6	0	0	++	0	0	0	0
1-03†	1530	1360	10	5	21	6315	118.9	3.2	0	5	+++	—	—	—	—
1-86†	2000	2010	6	5.5	21	6113	120.9	2.5	0	0	+	0	Few	0	0
7-1	2610	2510	10	5	25	6648	108.4	2.9	0	5	+	Few	"	0	0

9-0	3020	3020	10	11	25	10,751	147.0	3.8	0	61	+	Medium	"	Slight	0
1-00	2400	2040	10	9	25	8659	122.3	3.5	0	88	+	"	Medium	Marked	0
1-12	1760	1920	10	9	34	8091	107.4	0.8	0	0	++	0	Few	0	0
1-07	1720	2380	10	5	37	8659	130.1	1.4	0	3	++	Few	0	0	0
1-20	1820	2040	10	7	39	7284	105.4	0.3	0	0	++	"	Medium	0	0
3-3	2040	2160	5	7	47	8012	116.1	1.2	0	30	+	"	Few	0	0
3-6	1880	2080	2	3.5	47	9161	107.5	1.8	0	92	−	Many	"	0	0
1-02	1960	2330	10	5	53	7238	102.0	0.8	0	0	++	0	0	0	0
1-04	1720	2550	10	5	53	8659	123.8	2.3	0	20	++	0	0	0	0
9-4	1920	1920	3	3.5	63	8495	123.1	1.5	0	85	+	Medium	Few	0	0
9-3	2060	2480	1	7	93	6504	103.2	0.8	0	15	++	0	0	0	0
1-74†	1900	2080	6	7.5	149	6113	95.0	2.0	0	0	++	0	0	0	0
Controls	(4)‡	1505	—	—	—	5135	78	1.5	0	0	−	0	0	0	0
"	(40)	1815	—	—	—	5317	83	1.6	0	0	−	0	0	0	0
"	(11)	2135	—	—	—	6070	86	1.4	0	0	−	0	0	0	0

* + = 20 or less; ++ = 25 to 80; +++ = 90 or more erythrocytes per glomerular section.

† Died spontaneously.

‡ Number of animals studied.

Finally, it can be seen that the only rabbit which showed a decrease in the azofuchsin excretion (No. 1-00) is the only one which showed a marked fatty degeneration (see page 775).

DISCUSSION

1. The Nature of the Disease.—In a preliminary report (1937) it was stated that the changes produced resembled not so much those of diffuse hemorrhagic glomerular nephritis as those of focal glomerular nephritis as observed in bacterial endocarditis. At that time we had studied a few rabbits only, Nos. 3-3, 3-6, 9-4 and 9-3 of the present series. It is evident from Table IV that in these cases distinctly focal changes, such as adhesions, crescents and fibroses, were the predominant lesions. From the same table it can be seen, however, that in other animals, in addition to the focal changes which may or may not be present, there are what appear to be *diffuse* lesions, namely enlargement of all or nearly all the glomeruli, increase in the number of their nuclei and decrease in their blood content. These lesions now appear to be invariably present; but at the time the former animals were studied they had mostly disappeared. The question whether in the milder cases these lesions were also focal, *i.e.*, whether there were glomeruli or loops which were not affected (non-embolic focal glomerular nephritis), or whether the differences in our results were mainly differences in degree, some kidneys being more affected than others, we were unable to decide.

If we compare our findings with those obtained in man, we find a close resemblance. If we consider only such early cases in which the diagnosis has been secured anatomically as well as clinically, we discover that in man there are also two types of changes: (*a*) enlargement of the glomeruli, increase in the number of their nuclei and decrease in their blood; and (*b*) deposition of fibrin and crescent formation. Also in man the glomerular loops are swollen and fused. The capillaries contain a "protoplasmic" substance with an increased number of nuclei therein. The tufts are anemic. This change has been observed as early as 3 days after the clinical onset of the disease (Fahr, 1926). It appears to be typical of glomerular nephritis and invariably present (Gross, 1919; Fahr, 1925, 1926; Koch, 1927; McGregor, 1929; Volhard, 1931; Ehrich and Sommer, 1933; Fish-

berg, 1934; Bell, 1936, 1937). A deposition of fibrin, however, has been observed in some cases only, and if it was present, it was observed only in some glomeruli or in some isolated loops. Thus Gross saw hyaline thrombi but rarely. Fahr (1926) found no fibrin in his case. McGregor found fibrin occasionally in a few glomerular loops. Bell (1937) observed thrombi or crescents in 26 of 51 cases, but it appears that at least 5 of these cases should not be classified as glomerular nepbritis.

Turning to the functional changes, we wish to stress the close conformity of our findings with the earlier data obtained in rabbits and rats. In our rabbits oliguria was present beginning with the 7th day; Weiss (1935–36) and Tsuji (1936–37) observed it in the 2nd week and thereafter. In our rabbits, marked proteinuria was present from the 7th day onward; Masugi (1933–34) observed it after the 6th to 8th day, Hemprich (1935) after the 5th to 9th day, Weiss (1935–36) after the 7th to 12th day, and Tsuji (1936–37) after the 5th to 7th day. In our animals, blood was found in the tubules from the 6th to the 63rd day; Masugi (1933, 1934) found hematuria regularly; Tsuji (1936–37) observed it as early as in the first week, and in some animals a long time after the acute phase of the disease. It is true that in rats Smadel and Farr (1936, 1937) found no significant hematuria, if they injected a pure nephrotoxin; but in these cases the histological appearance was not typical, since according to their own description proliferation and infiltrative changes were practically absent. Nitrogen retention was found in the 2nd week in our rabbits. Masugi (1934) found it also in rabbits which recovered later. Since all these findings conform very closely, not only in their character, but also in their time of occurrence, we feel entitled to believe that our data are not casual findings, but represent a true cause and effect. Since it has further been demonstrated (Masugi, 1934; Smadel and Farr, 1937) that these animals may also show hypertension, fall in blood urea clearance, fall in the plasma proteins, lipemia and lipuria, it appears that, anatomically as well as functionally, our disease is the same as human diffuse hemorrhagic glomerular nephritis.

2. Pathogenesis.—Turning to the pathogenesis of the disease, it should be noted that there was, as partly observed by Masugi (1933),

Hemprich (1935), Weiss (1935–36) and Tsuji (1936–37), a period of latency of about 1 week before the clinical symptoms became manifest. It is true that there was in some cases edema and oliguria the day following the introduction of the serum; but the diuresis quickly returned to normal and in most cases tested during this period there was little or no protein in the urine. We believe, therefore, that this primary reaction was the result of a general disturbance, perhaps resulting in a greater capillary permeability and consequent edema and oliguria. That such a latency may also occur in man, is mentioned by Fahr (1925) and Volhard (1931). The latter also cites Kylin's observation that in cases of scarlatina a rise in capillary pressure occurred, even several days to 1 week before the nephritic symptoms became manifest.

It should be noted also that in 4 of our 5 animals tested between the 3rd and 7th days, the urine volume was conspicuously above the normal average. Furthermore, and this has also been observed by Fahr (1934), Hemprich (1935) and Tsuji (1936–37), in most of the animals which died or were killed during this period there was hyperemia of the glomeruli. On the other hand, the glomeruli were anemic only from the 6th day onward when the glomerular changes were already well developed. It must therefore be concluded that nephrotoxic glomerular nephritis in rabbits does not start with arteriolar spasm, but that the glomeruli become anemic only when the loops are clotted with protoplasmic material.

The first morphologic changes observed were, as has already been stated, an enlargement of the glomeruli and an increase in the number of their nuclei. This increase was fully developed on the 7th to 8th day (Table IV). Fahr (1935) states that he saw the endothelial proliferation as early as on the 4th to 5th day, and Tsuji (1936–37) on the 4th to 8th day, while Masugi (1933) claims that it can be found in rats after 24 hours. It appears, therefore, that proliferation starts soon after the injection of the serum and reaches a visible or measurable amount in the second half of the first week. Since, just as in man (Gross, 1919; McGregor, 1929) we found abundant mitoses during this period, it is evident that the increase of cells was due to a multiplication of local cells. The question whether these cells were endothelial cells, as maintained by most authors, or

whether they were elements outside the capillaries (MacCallum, 1934) or blood cells, as has been suggested, is one that we could not decide.

As to the cause of the multiplication of the cells, it is true that we found pyknotic nuclei in the glomeruli of a few rabbits during the first week, as did Tsuji (1936–37) in rabbits, and Gross (1919), Fahr (1925), Koch (1927) and McGregor (1929) in man. But as they were present in only a few instances, it appears unlikely that necrohormones were the sole cause of the proliferation. We believe rather that the leucocytes present, which incidentally have been observed by all students of acute glomerular nephritis, were the main source of the growth promoting substances.[2] This view seems to be supported by the frequent observation of disintegrating polymorphonuclear leucocytes within the tissue of the loops.

The second lesion, namely the deposition of fibrin, was in our series first observed 8 days after the injection of the serum. Hemprich (1935) observed it in rabbits on the 7th day, and Masugi (1933) in rats as early as the first day. As to the location of the fibrin, we were unable to convince ourselves that there were intracapillary thrombi. In our experience, the fibrin is located outside of the capillaries, either between the loops or between them and the capsule. It can be seen from Table IV that the fibrin disappears gradually within the 2nd week, and that adhesions, crescents and fibroses develop in its place. It is also evident that adhesions, crescents and fibroses develop only in those glomeruli in which fibrin had previously been deposited. It follows, therefore, that crescents are not an essential feature of glomerular nephritis, but that, as in focal embolic nephritis or malignant nephrosclerosis, they represent a complication. That this holds also in man is a common experience. It is for this reason that Fahr (1925) distinguishes between intra- and extracapillary nephritis. But if the formation of crescents is merely a complication, we can no longer look upon it as being pathognomonic of the subacute phase of the disease, as is commonly done. That this is an erroneous idea is obvious also from the fact that in man (Koch, 1927), as in rabbits, crescents are found as early as in the 2nd week of the disease.

[2] The increase in leucocytes has also been demonstrated clinically (Addis and Oliver, 1931; Murphy, Grill and Moxon, 1934, and others).

The cause of the deposition of fibrin has not been detected. It is evident from Tables I and IV that it is not due to the introduced serum or to the size of the dose alone. Rabbits 1-00 and 1-12, for example, both receiving the same dose of the same serum, reacted very differently in this respect, rabbit 1-00 showing that 88 per cent of the glomeruli contained fibrin, rabbit 1-12 none. In rats Masugi (1933) found fibrin only when he injected large doses, and Smadel (1936, 1937), also using rats, found it either as a result of giving a large amount of a relatively pure nephrotoxic serum or a smaller amount of serum rich in non-organ-specific anti-rat-tissue antibodies as well as in the more specific nephrotoxin. At least in rabbits, then, the kind or amount of serum is not the sole factor, but individual susceptibility appears to play an important rôle. Whether the deposition can be taken for an anaphylactoid reaction, as Smadel suggests, cannot as yet be decided. If this were true, we must conclude that such a reaction occurs also in man, since fibrin and crescents are also seen in human glomerular nephritis. However, it appears certain that this lesion amounts to much more than proliferation alone. The old idea of Loehlein, which is held also by Fishberg (1934), seems to be correct, namely that crescents are an indication not so much of the subacute stage, as of a stormy course of the disease.

Concerning the outcome of our disease, it appears that Masugi (1933) is right when he assumes that glomeruli that are simply enlarged and have an increased number of nuclei can return completely to normal. The question whether destruction of the glomeruli is a result of fibrin deposition only, or whether there is also a hyalinization of the loops following pure proliferation, cannot be answered from our material.

As to the tubular changes, it has been demonstrated (page 779) that the fatty changes follow the deposition of fibrin. Both can therefore be attributed to the same factor, as has been maintained by Fahr (1925). It should be noted that after the 4th week no more fat was seen (Table IV). Instead, we found granules in the epithelial cells the nature of which could not be detected.

3. *Correlation of Morphology and Function.*—The attempt to correlate certain functional changes with the lesions found in our kidneys produces some definite relationships. It has been demonstrated

already (page 779) that the onset of marked proteinuria and oliguria occurs at the time when the glomerular changes have reached full development. It has further been demonstrated (Table IV) that during the period of proteinuria and oliguria the output of the glomerular dye, cyanol, is markedly reduced, whereas the excretion of the tubular dye, azofuchsin, with one exception was not changed.

The proteinuria seems without doubt to be caused by the glomerular damage. The initial oliguria may be said to be due to extrarenal factors, both because edema was present and because at this time there were no glomerular or tubular changes which would explain a diminished diuresis. The oliguria in the 2nd week and thereafter, however, can hardly be explained by such an assumption, as there had been a period of good diuresis or polyuria in between. As the filtering membrane was very much thickened during the period of oliguria, and as there was a marked anemia of the glomeruli at this time, and as in rabbits diuresis is largely regulated by the glomeruli (Kaplan and Smith, 1935), it appears that Fahr (1925) was probably right when he concluded that oliguria in acute glomerular nephritis (other than the initial oliguria) is due rather to glomerular damage than to extrarenal factors.

As to the decrease in cyanol excretion, it has been demonstrated (page 774) that it cannot be explained by the oliguria alone. Nor did the dye escape into the edema fluid, as in no case was any dye seen therein. It is also unlikely that some dye diffused back from the lumina of the tubules into the blood or lymph stream through possibly damaged epithelial cells, as in this case it would be difficult to understand why the excretion of azofuchsin was not diminished. However, it might be possible that the proportion of the water which is excreted extrarenally was increased in these animals, and that some dye left the plasma with this water. It might also be that a larger amount of dye was adsorbed by the thickened filtering membrane. However, as water diuresis in rabbits appears to be both a glomerular and a tubular function, we favor the explanation that the oliguria was mainly caused by hindering the glomerular contribution to diuresis. If the tubules functioned more or less normally, it should be expected that the decrease in cyanol output was greater than the decrease in diuresis. Though we are unable to

prove at present that this explanation is correct, it is clear from our findings that the retention of cyanol parallels the oliguria and the morphological changes in the glomeruli. It appears, therefore, that, if the oliguria is mainly a result of glomerular damage, the retention of cyanol is due to the same cause.

In the only test performed during the first week of the experiment the diuresis and the dye excretion were both increased. As the amount of dye excreted corresponded to the degree of diuresis, it appears that filtration was actually increased during this time.

The azofuchsin excretion, as has been pointed out (page 775), with but one exception was undisturbed. In all these animals the tubular epithelial cells were little if any changed, and the only animal (rabbit 1-00) which showed a diminished azofuchsin output was also the only one which showed a marked fatty change. It therefore appears obvious that the fundamental question which has been mentioned in the introduction, namely whether glomeruli and tubules may function independently of each other, must be answered affirmatively.

SUMMARY AND CONCLUSIONS

It has been shown in this paper that structural and functional changes in acute glomerular nephritis in rabbits produced by nephrotoxins by the method of Masugi are the same as those found in human glomerular nephritis. The morbid anatomy is characterized by glomerular cell proliferation, and in some cases by deposition of fibrin and crescent formation of the glomeruli and by fatty changes of the tubules. The functional changes are: oliguria, proteinuria, hematuria, cylindruria, edema, rise in blood urea, and according to Masugi (1933, 1934) and Smadel (1936, 1937), rise in blood pressure, lipuria, and fall in urea clearance and plasma proteins. As we are unaware of any discrepancies between the experimentally induced disease and human nephritis, the conclusion follows that the two so closely resemble each other that they appear to be identical.

As to the pathogenesis, it has been shown that the disease begins with a period of latency. This is characterizied anatomically by hyperemia of the glomeruli; and functionally, in at least a number of cases, by an increased diuresis. It follows, therefore, that the theory of Volhard, according to which glomerular nephritis is caused by

arteriolar spasms, can no longer be maintained. It has further been demonstrated that proliferation of glomerular cells is the typical lesion, and that deposition of fibrin and crescent formation occur only in certain cases, and in these only in a widely varying number of glomeruli. As crescents are found as early as the proliferation itself, it follows that they should not be regarded as pathognomonic of the subacute phase, but that they represent a complication which probably aggravates the disease.

As to correlation of morphological and functional changes, it has been demonstrated that oliguria, marked proteinuria and diminished excretion of cyanol appear at the time when the glomerular changes are at their peak. Evidence has been presented that the oliguria and the decrease in cyanol excretion in acute glomerular nephritis are chiefly the result of the glomerular damage. It has further been demonstrated that the excretion of azofuchsin was unchanged, except for a diminution in the rabbit which at autopsy showed a marked fatty change of the tubules. We regard these observations as evidence, that, in acute glomerular nephritis in rabbits, glomeruli and tubules may function independently of each other.

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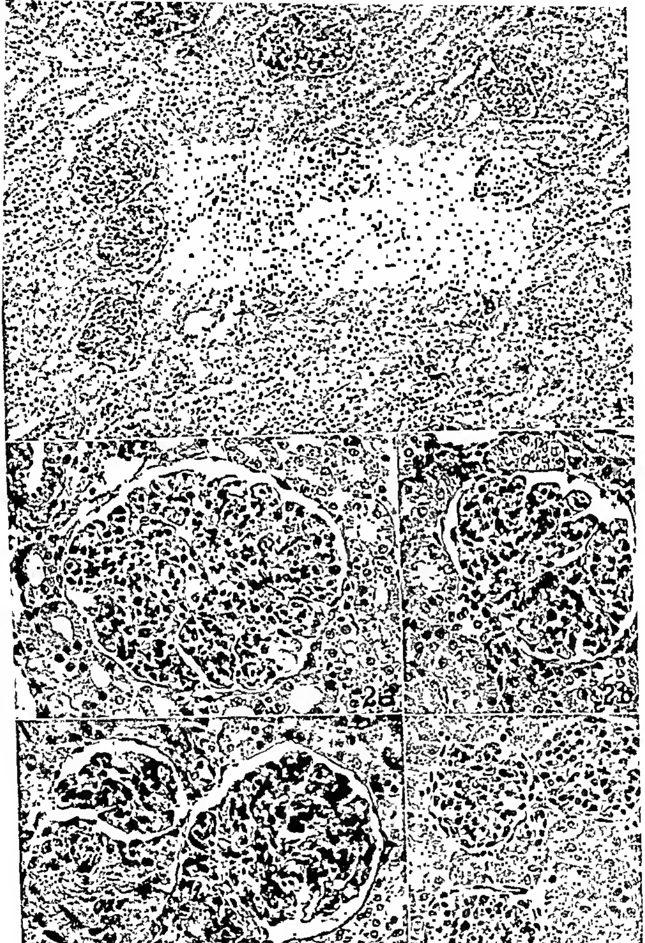
EXPLANATION OF PLATES

Plate 32

Fig. 1. Rabbit 1-70. Diffuse glomerular nephritis of 8 days' duration. The glomeruli are much enlarged and rich in nuclei. Hematoxylin-eosin. × 110. (Compare with Fig. 2*c*, the magnification of which is more than double that of Fig. 1.)

Fig. 2. (*a*) Rabbit 1-19; nephritis of 8 days' duration; and (*b*) Rabbit 1-21; nephritis of 10 days' duration. Glomeruli showing increase in size and in the number of their nuclei. Hematoxylin-eosin. × 240. (*c*) Rabbit 1-92. Normal glomeruli. Hematoxylin-eosin. × 240. (*d*) Rabbit 1-21; nephritis of 10 days' duration. Glomeruli showing deposition of fibrin between loops and capsule. Azur II-eosin. × 240.



(Ehrich *et al* : Acute glomerular nephritis)

PLATE 33

FIG. 3. Rabbit 1-00. Nephritis of 25 days' duration. Capsular adhesions and crescents. Azur II-eosin. × 240.

FIG. 4. Rabbit 1-00. Nephritis of 25 days' duration. Glomerulus showing typical crescent. Azur II-eosin. × 240.

(Ehrich *et al.*: Acute glomerular nephritis)

Plate 34

Fig. 5. Rabbit 7-0. Nephritis of 12 days' duration. Hyaline casts with dilatation of capsular spaces. Azur II-eosin. × 110.

Fig. 6. Rabbit 7-0. Nephritis of 12 days' duration. (*a*) Erythrocytes and protein in capsular space. × 240. (*b*) Erythrocytes in tubule. × 480. Azur II-eosin.

(Ehrich *et al*: Acute glomerular nephritis)

PLATE 35

FIG. 7. Rabbit 7-0. Nephritis of 12 days' duration. Dilatation of tubules and glomerular spaces. Note the proliferation in the glomeruli. Azur II-eosin. × 110.

FIG. 8. Rabbit 1-00. Nephritis of 25 days' duration. Marked fatty change of the distal portion of the proximal convoluted tubules. Hematoxylin-Sudan. × 240.

FIG. 9. Rabbit 9-7. Nephritis of 12 days' duration. Hyaline droplets in convoluted tubules. Azur II-eosin. × 480.

(Ehrich *et al*: Acute glomerular nephritis)

THE EFFECT OF A TISSUE ENZYME UPON PNEUMOCOCCI

By RENÉ J. DUBOS, Ph.D., and C. M. MacLEOD, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research*)

(Received for publication, February 2, 1938)

Rabbits immunized by the intravenous injection of heat-killed encapsulated pneumococci respond by the production of the type specific antibodies directed against the capsular polysaccharide of the cell used as antigen. When, however, the same antigen is injected into animals of the same species by the intradermal route (skin of the mid-flank) the type specific antibodies fail to appear (1-3). It appeared possible that when pneumococci are injected into the skin of the rabbit, they are subjected to the action of certain tissue enzymes which inactivate the type specific antigen. It is known in fact that this antigen is readily inactivated by the autolytic ferments of the bacterial cell (4, 5); these ferments however, are completely destroyed by heating in the course of preparation of the bacterial antigen. The present paper reports the existence of an enzyme, widely distributed in animal tissues, which modifies the staining properties and the antigenicity of heat-killed pneumococci.

EXPERIMENTAL

Cultures.—Virulent pneumococci were grown and preserved in blood broth and passed through mice often enough to maintain a degree of virulence such that 0.000,000,01 cc. of an 8 hour culture would kill 20 gm. mice within 72 hours. Cultures of R variants were similarly grown in blood broth.

Heat-Killed Cells.—Pneumococci recovered from a young broth culture were resuspended in a small amount of distilled water and rapidly added to a larger volume of distilled water at 75°C.; the temperature was maintained at this level for 20 minutes. This process of "flash" heating was selected because it minimizes the chances of alterations due to autolytic action (6).

Method of Immunization.—The cell suspensions were injected into rabbits intravenously on 6 consecutive days followed by a free interval of 1 week. Three courses were given, each animal receiving daily a dose containing the cells from 1 cc. of broth culture. The rabbits were bled on the 8th day after the last injection of each course.

Serological Tests.—In all precipitin reactions, 0.5 cc. of immune serum or 0.2 cc. of serum diluted to 0.5 cc. with salt solution, was added to an equal volume of graded dilutions of the purified capsular polysaccharide. The mixtures were incubated for 2 hours at 37°C.; final readings were made after keeping the reacting mixtures overnight in the ice box.

Preparation of the Enzyme.—Methods of extraction of the enzyme from polymorphonuclear leucocytes are described in the present paper. The purification of the enzyme extracted from dried pancreatin, and determination of its chemical activity, have been described elsewhere (7).

The Fate of Heat-Killed Pneumococci Injected into the Skin of the Rabbit.—As stated above, heat-killed encapsulated pneumococci injected into the skin of the rabbit fail to stimulate the formation of the type specific carbohydrate antibodies which arise in response to the intravenous injection of the same antigen. An attempt was therefore made to follow by microscopic examination the fate of pneumococci injected into the skin, in the hope of finding a clue to the mechanism of lack of antigenic response.

Experiment 1.—0.1 cc. of a suspension of heat-killed pneumococci (Type I) was injected at several sites into the skin of a rabbit. The injected areas were excised at different intervals of time, and films, made from the tissue fragments, were stained by the Gram technique.

There was, of course, a pronounced polymorphonuclear infiltration at the sites of injection, but at first only little, if any, phagocytosis. The pneumococci, however, were seen to undergo a process of extracellular digestion which began within 24 hours after injection, and was completed in 4 to 5 days; many bacteria became Gram-negative before being engulfed by the leucocytes. These morphological observations suggested that the pneumococci were being attacked by ferments released at the site of injection.

The Effect of Leucocytic and Other Tissue Enzymes on the Morphology of Heat-Killed Pneumococci.—Normal rabbit skin was ground up, then extracted under a wide range of conditions in an attempt to separate the principle responsible for the change in staining reaction of the injected pneumococci (Experiment 1).

The extracts of normal skin exhibited marked enzymatic activity when tested with a number of soluble substrates (proteins, phosphoric esters, fats, etc.) but completely failed to exert any appreciable action on the bacterial cells.

The presence of an inflammatory reaction at the site of inoculation suggested that leucocytes released soluble ferments capable of attacking the pneumococci. A number of experiments were instituted to determine the validity of this assumption (Experiment 2).

Experiment 2.—A sterile, polymorphonuclear exudate was obtained by injecting aleuronate into the pleural cavity of a normal rabbit. The exudate, consisting almost exclusively of polymorphonuclear cells, was collected after 24 hours. The cells were washed, resuspended in saline, allowed to autolyze at 37°C. for 48 hours, then extracted with N/10 acetic acid for 24 hours. The extract was heated at boiling temperature for 10 minutes and the coagulum removed by centrifugalization; the new precipitate which formed on neutralization of the supernate was also discarded. The material was then dialyzed, and again centrifuged; the clear soluble fractions were used as enzyme. 0.1 cc. of purified leucocytic extract was added to 1 cc. of a suspension of heat-killed pneumococci at pH 7.0; the mixture was incubated at 37°C. for 24 hours. Films made from this preparation were stained with the eosin-methylene blue stain or by the Gram technique.

Whereas the control untreated pneumococci were intensely stained by methylene blue, and reacted positively to the Gram stain, the cells treated with the leucocytic extract had lost all affinity for the basic dyes and appeared as faint red structures when counterstained with eosin or safranin. The cell bodies, however, were not disintegrated but on the contrary retained their characteristic morphology. It is also worth noting that there was no other evidence of lysis, since the bacterial suspension lost only little of its original turbidity.

The same changes in staining characters can be observed when R or S variants of pneumococci, irrespective of type derivation, are treated with the leucocytic extract. The results are also identical when the bacterial cells are killed by methods other than heating, such as treatment with formaldehyde and acetic acid.

Similar extracts, capable of destroying the affinity of pneumococci for the basic dyes, but without causing any disintegration of the cell structure, have been obtained from the organs—in particular the liver, pancreas, spleen and lungs—of several animal species. The same enzyme has also been prepared from the pleural exudate of a tuberculous patient, who developed empyema following injection with *Hemophilus influenzae*.

All the enzyme preparations have a number of properties in common, irrespective of the source from which they are obtained. The

active principle is heat resistant, especially at slightly acid reactions; its rate of activity upon pneumococci increases with temperature up to 70°C.; its range of activity is between pH 5.0 and pH 9.5; it is completely resistant to trypsin and chymotrypsin, but is rapidly inactivated by pepsin; it gives the common protein reactions. Finally all the preparations exhibit a high degree of enzymatic activity on yeast nucleic acid.

It may be noted that all these properties are the same as those of the purified nuclease (prepared from dried pancreatin) which has been described elsewhere (7). In fact, the purified nuclease from this source is also capable of attacking pneumococci, giving rise to the same alterations in staining reactions brought about by the leucocytic enzyme described above.

Effect of the Leucocytic Enzyme on the Antigenicity of Encapsulated Pneumococci.—Heat-killed encapsulated pneumococci, injected into rabbits by the intravenous route, elicit the production of the specific antibodies directed against the capsular polysaccharide of the cell used as antigen. The effect of the leucocytic enzyme on this "capsular antigen" is determined in the following experiment.

Experiment 3.—A purified extract obtained from the pleural exudate of a tuberculous patient who had developed empyema following secondary infection with *H. influenzae* was prepared according to the method described in Experiment 1. 10 cc. of this extract (at pH 7.0) were added to an equal volume of suspension of heat-killed Type I pneumococci. The mixture was incubated at 37°C. for 38 hours. Some of the bacterial suspension diluted with saline at pH 7.0 without the addition of active enzyme was also incubated to serve as control. Both suspensions were centrifugalized at the end of the incubation period, and the cells resuspended in saline were used for the immunization of two groups of 3 rabbits each by means of the intravenous route. The animals were bled after the third course of immunization and their sera were tested for the presence of type specific precipitins and agglutinins.

Stained films of the two bacterial suspensions used for immunization showed the control cells to be strongly basophilic and Gram-positive whereas over 95 per cent of the cells treated with enzyme had lost their affinity for the basic dyes; the treated cells, however, did not show any evidence of cellular disintegration or lysis.

The 3 rabbits immunized with the Gram-positive cocci developed in their sera precipitins for the Type I polysaccharide; type specific

agglutinins also appeared in respective titers of 1:320, 1:80, 1:80. Of the 3 rabbits immunized with the enzyme-treated cells, one showed in its serum weak precipitins for the Type I polysaccharide; the other 2 were negative. The type specific agglutinins were also much reduced, the titers being respectively 1:20, 1:2, 1:2.

Five similar experiments were performed. In all cases there was marked reduction, or complete absence, of the type specific precipitins and agglutinins in the sera of animals immunized with the enzyme-treated cells, as compared with the sera of animals immunized with control encapsulated pneumococci. It was, however, found impossible to obtain bacterial suspension in which, after treatment with the enzyme, all the cells had lost their basophilic property. A small percentage of intact cells persisted in all cases and these cells probably accounted for the slight residual type specific antigenicity of the bacterial suspensions treated with the leucocytic enzyme, since it is known that heat-killed cells of Type I pneumococci constitute an antigen active in minute amounts.

Nature of the Action of the Leucocytic Enzyme on Pneumococci.—It has been shown that the action of the leucocytic enzyme on pneumococci can be recognized by two types of alteration: (*a*) destruction of the basophilic properties of the bacterial cell; (*b*) inactivation of the capsular polysaccharide antigen. A complete understanding of these findings depends of course on a knowledge of the chemical nature of the substrates attacked by the enzyme. The enzyme, although it inactivates the capsular polysaccharide antigen does not attack the capsular polysaccharide itself. In fact, of all of the soluble substrates tested, yeast nucleic acid is the only one to be attacked by the purified preparations. An analysis of this reaction is presented elsewhere (5).

The Gram-positive structure of heat-killed pneumococci is completely resistant to crystalline pepsin, but it is slowly attacked by some preparations of crystalline trypsin and chymotrypsin. It was found, however, that the same preparations of crystalline trypsin or chymotrypsin which slowly reduce the basophilic properties of pneumococci also exhibit some enzymatic activity against yeast nucleic acid. With repeated recrystallizations of the proteolytic enzymes, however, the activity against pneumococci and nucleic acid eventually

disappears, although the proteolytic activity remains unimpaired.[1] It is evident therefore that the loss of the basophilic character of the cell is not associated with proteolysis. These observations also emphasize once more the close correlation which exists between the ability of the enzyme preparations to decompose yeast nucleic acid and to attack the bacterial cell.

DISCUSSION

It is possible to extract from washed polymorphonuclear leucocytes, and from many animal tissues, an enzyme capable of destroying the basophilic character of heat-killed pneumococci, and of inactivating the capsular polysaccharide antigen of encapsulated cells of the same species. The enzyme, however, does not decompose the capsular polysaccharide itself, and since it attacks both R and S variants, the point of attack of the bacterial cell by the enzyme must be a structure common to all pneumococci. Of all soluble substrates tested, yeast nucleic acid is the only one to be decomposed by the purified enzyme preparations; it is also true that a purified nuclease obtained from dried pancreatin attacks both the basophilic structure and the capsular antigen of pneumococci. It is therefore possible that the cellular structure attacked by the enzyme is related to nucleic acid; this point of view will be substantiated by a description to be presented in a later publication of the products released in solution when the enzyme attacks the bacterial cell. No explanation is available at the present time of the mechanism whereby the encapsulated cell which loses its basophilic character, loses at the same time its effectiveness as type specific antigen. It is worth repeating in this connection that the enzyme does not cause any lysis or disintegration of the cell body, nor does it decompose the capsular polysaccharide.

The question which prompted the present study was to account for the failure of rabbits to develop type specific antibodies following the intradermal injection of heat-killed encapsulated pneumococci. It was observed that dead pneumococci injected into the skin remain at the site of injection for several days and bring about an accumulation of polymorphonuclear leucocytes; it was also demonstrated that these

[1] We are greatly indebted to Dr. J. H. Northrop and Dr. M. Kunitz for supplying samples of crystalline trypsin and chymotrypsin recrystallized several times.

leucocytic cells contain an enzyme which inactivates the capsular antigen *in vitro*. The change in staining reaction of the injected pneumococci suggests that the same enzymatic action may take place *in vivo* and account, partly at least, for the lack of type specific antigenic response to the intradermal injection of the bacterial cells.

SUMMARY

Polymorphonuclear leucocytes contain an enzyme which destroys the basophilic character of heat-killed pneumococci (R and S variants) and inactivates the type specific polysaccharide antigen of encapsulated cells. The same enzyme, however, fails to cause a disintegration of the bacterial cells, or to decompose the capsular polysaccharide itself.

The enzyme has been extracted from a number of animal tissues; it appears identical with a purified enzyme extracted from pancreatin and which decomposes yeast nucleic acid.

These facts are considered with regard to the failure of rabbits to produce the type specific carbohydrate antibodies when immunized with heat-killed encapsulated pneumococci by the intradermal route.

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IMMUNIZATION OF EXPERIMENTAL ANIMALS WITH A SOLUBLE ANTIGEN EXTRACTED FROM PNEUMOCOCCI

By RENÉ J. DUBOS, Ph.D.

(*From the Hospital of The Rockefeller Institute for Medical Research*)

(Received for publication, February 2, 1938)

Pneumococci undergo, during autolysis, a change from the Gram-positive to the Gram-negative state. This change in staining reaction need not be accompanied by any gross lysis or disintegration of the cell structure; indeed, suspensions of pneumococci can be obtained which no longer take the Gram stain but retain otherwise their characteristic morphology (1, 2). It has been shown, on the other hand, that encapsulated pneumococci which have become Gram-negative under these conditions, lose the capacity to stimulate the production of the type specific capsular antibodies when injected into rabbits (3). It is of obvious interest for an understanding of the Gram reaction, and for a study of the antigenic structure of pneumococci, to determine the nature and properties of the substances released in solution when these bacterial cells become Gram-negative. The chemical nature of these substances will be described in other papers. It is shown in the present study that rabbits and mice injected with this soluble fraction develop immunity against infection with virulent pneumococci.

EXPERIMENTAL

Cultures.—Virulent pneumococci were grown and preserved in blood broth and passed through mice often enough to maintain a degree of virulence such that 0.000,000,01 cc. of an 8 hour culture would kill 20 gm. mice within 72 hours. Cultures of R variants were similarly grown in blood broth.

Immunization and Immunity Tests.—The route of immunization and the amount of antigen used are described for each experiment.

The rabbits were bled one week after they had received the last immunizing dose. Their sera were tested for the presence of protective antibodies by the intraperitoneal injection into white mice of mixtures of 0.5 cc. of serum and of virulent cultures diluted in 0.5 cc. of meat infusion broth.

White mice were tested for active immunity one week after they had received the last immunizing dose. The infecting dose was given by the intraperitoneal route.

Different techniques have been described in preceding papers for the preparation of suspensions of pneumococci which become Gram-negative without undergoing cellular disintegration (1–3). It has been shown in particular that pneumococci resuspended in organic acids at pH 4.0–4.4 remain Gram-positive as long as they are maintained at acid reactions. If, however, the same cell suspension is later neutralized, an enzymatic action takes place which changes the bacterial cells from the Gram-positive to the Gram-negative state, without causing further autolysis. The soluble products released during this change have been used in the following experiments for the immunization of rabbits and mice.

The Immunization of Rabbits with a Soluble Antigen Prepared from Type I Pneumococci. Experiment 1.—

The cells from 3 liters of a 4 hour culture of virulent Type I pneumococci were separated by centrifugalization and resuspended in 60 cc. of M/10 acetate buffer at pH 4.2. The acid suspension of bacterial cells was kept at 37°C. overnight. The cells, which were still Gram-positive, were centrifugalized the next day, and resuspended in 35 cc. of M/20 phosphate buffer at pH 7.2. The neutral bacterial suspension was incubated at 37°C. After 6 hours incubation, the cells had become Gram-negative, but there was no evidence of lysis. The cells were then centrifugalized, the supernatant fluid filtered through a Berkefeld filter (V) and the filtrate used for the immunization of 3 rabbits. Each rabbit received 5 cc. of the filtrate on 2 consecutive days.

A similar antigen preparation was obtained one week later from 5 liters of broth culture of Type I pneumococci and the 3 rabbits again received 5 cc. of filtrate on 2 consecutive days.

The animals were bled one week after the last immunizing dose and a pool of the 3 sera was tested in mice for the presence of protective antibodies. The mice were infected with different dilutions of virulent cultures of pneumococci of Type I (SVI) and of Type III (A/66/S). The results are presented in Table I.

The results of Experiment 1 show that the serum of rabbits immunized with the filtered antigen obtained from Type I pneumococci was able to protect mice against 100,000 fatal doses of virulent Type I pneumococci and against 1000 doses of Type III pneumococci.

Properties of the Soluble Antigen Extracted from Type I Pneumococci.—The filtered antigen obtained from Type I pneumococci by the method described in Experiment 1 gives a distinct precipitate when treated with acetic acid at pH 4.2. This precipitate is readily soluble at neutral reaction; the neutral solution can be heated at boiling temperature without giving any evidence of coagulation. The effect of acid precipitation and of heating on the antigenicity of this fraction is considered in Experiment 2.

Experiment 2.—The antigen was prepared from 16 liters of broth culture of Type I pneumococci according to the method described in Experiment 1. The

TABLE I

The Protective Action in Mice of the Serum of Rabbits Immunized with a Soluble Fraction of Type I Pneumococci

Pooled sera of rabbits immunized with soluble antigen	Infecting pneumococci						
	Amount of culture	Type I			Type III		
cc.	*cc.*						
0.5	10^{-3}	S	S	S	—	—	—
	10^{-4}	D 72	S	S	D 72	D 72	D 72
	10^{-5}	S	S	S	D 72	S	S
	10^{-6}	S	S	S	S	S	S
	10^{-7}	—	—	—	S	S	S
0 (control mice)	10^{-6}	D 72			D 22		
	10^{-7}	D 72			D 72		
	10^{-8}	D 72			D 72		

S = survival of the animal.

D = death of the animal; the numeral indicates the number of hours before death.

filtered solution was treated with acetic acid at pH 4.2; the precipitate was separated by centrifugalization and finally put back in solution in 150 cc. of neutral saline solution. Half of the solution (75 cc.) was kept at 0°C., and the other half heated to 80°C. for 10 minutes. The two fractions were used for the immunization of 2 groups of 3 rabbits each according to the method described in Experiment 1. The sera were tested in mice for the presence of protective antibodies effective against pneumococci of Type I (SVI) and Type II (D39S). The results are presented in Table II.

The results presented in Table II show that the sera of the 3 rabbits immunized with the antigen precipitated at pH 4.2 protected mice

against infection with many fatal doses of Type I and Type II pneumococci. The sera of the 3 rabbits immunized with the antigen heated at 80°C. were much less effective. These 3 animals received an additional course of immunization with a new preparation of heated

TABLE II

The Protective Action in Mice of the Sera of Rabbits Immunized with the Soluble Antigen of Type I Pneumococci Precipitated with Acetic Acid or Heated at 80°C.

Immune rabbit serum		Infecting pneumococci				
Antigen used for immunization of rabbits	Rabbit No.	Amount of culture	Type I		Type II	
		cc.				
Antigen precipitated at pH 4.2	1	10^{-5}	S	S	S	S
		10^{-6}	S	S	S	S
	2	10^{-5}	S	S	S	S
		10^{-6}	S	S	S	S
	3	10^{-5}	S	D 72	S	S
		10^{-6}	S	D 42	S	S
Antigen heated at 80°C.	4	10^{-5}	D 42	D 96	D 48	S
		10^{-6}	D 96	D 120	S	S
	5	10^{-5}	D 72	D 120	D 48	D 48
		10^{-6}	D 72	D 72	D 42	S
	6	10^{-5}	D 72	D 96	D 48	S
		10^{-6}	D 96	D 96	S	S
Control mice		10^{-6}	D 42		D 42	
		10^{-7}	D 42		D 42	
		10^{-8}	D 42		D 120	

S = survival of the animal.

D = death of the animal; the numeral indicates the number of hours before death.

antigen; a marked increase in protective titer was then observed, especially against Type I pneumococci. It is apparent therefore that heating at 80°C. for 10 minutes did not completely destroy the immunizing power of the soluble antigen. However, a final answer to the question of heat stability of the antigen will require the immuniza-

tion of a large number of animals, since individual rabbits vary greatly in their response to the injection of the same preparation of antigen (Experiment 4).

Immunization of Rabbits with a Soluble Antigen Extracted from R Pneumococci.—The results of Experiments 1 and 2 indicate that the soluble antigen extracted from Type I pneumococci stimulates in rabbits the production of protective antibodies which are effective

TABLE III

The Protective Action in Mice of the Sera of Rabbits Immunized with a Soluble Extract Prepared from R Pneumococci

Immune rabbit serum		Infecting pneumococci			
Amount	Rabbit No.	Amount of culture	Type I	Type II	Type III
cc.		*cc.*			
0.5	1	10^{-5}	D 46 D 46	S S	— —
		10^{-6}	D 26 D 96	S S	D 48 D 48
		10^{-7}	D 96 S	S S	D 72 D 48
0.5	2	10^{-5}	S S	S S	— —
		10^{-6}	S S	S S	D 48 D 48
		10^{-7}	S S	S S	S S
0.5	3	10^{-5}	S S	S S	— —
		10^{-6}	S S	S S	D 48 D 48
		10^{-7}	S S	S S	D 48 S
0 (Control mice)		10^{-6}	D 46	D 46	D 46
		10^{-7}	D 46	D 46	D 46
		10^{-8}	D 96	D 46	D 96

S = survival of the animal.

D = death of the animal; the numeral indicates the number of hours before death.

not only against pneumococci of homologous type, but also against heterologous types. It has also been shown elsewhere that the change from the Gram-positive to the Gram-negative state (under the conditions described in Experiment 1) is a reaction which is common not only to all types of pneumococci, but also to the R as well as the S variants. It appeared therefore possible that a soluble antigenic fraction could be prepared from R pneumococci. The

immunization of rabbits with the fraction released in solution when R pneumococci become Gram-negative is illustrated in Experiment 3.

Experiment 3.—The culture used was an R variant derived from Type II Pneumococcus (D39R); 0.5 cc. of an 8 hour broth culture of this strain failed to kill a mouse when injected by the intraperitoneal route. The antigen was prepared from 5,000 cc. of a 4 hour broth culture, according to the method described in Experiment 1. The filtered soluble fraction (35 cc.) was injected intravenously into 3 rabbits on 3 consecutive days. The 3 rabbits were bled one week after the last immunizing dose and their sera were tested in mice for protective action against virulent pneumococci. The results are presented in Table III.

The results of Experiment 3 show that the sera of rabbits immunized with a soluble antigen prepared from R pneumococci protect mice against many fatal doses of virulent Type I and Type II pneumococci; there was only a very slight degree of protection against Type III pneumococci.

Studies on the technique of preparation of the soluble antigen would be rendered easier if there were available a rapid method for the comparison of the antigenic efficacy of different extracts. It is shown in the following experiment that it is indeed possible to obtain a positive antigenic response to the injection of a single dose of antigen into rabbits.

Experiment 4.—The soluble antigen was prepared from 30 liters of a broth culture of R pneumococci (derived from Type II) by the method described in Experiment 1. The filtered solution was precipitated with acetic acid; the precipitate was redissolved in 50 cc. of saline by careful addition of dilute NaOH. The antigen was administered to 10 rabbits in a single injection of 5 cc. per rabbit. The animals were bled one week later and their sera were tested in mice for protective action against Type I pneumococcus.

The results presented in Table IV show that certain rabbits respond to the injection of a single dose of antigen with the production of protective antibodies. The marked individual variations unfortunately make it difficult to compare the antigenic value of different preparations unless large numbers of animals are used. Experiments are now in progress to increase the protective titers of the immune sera. It has been found, however, that the rabbits which fail to give a definite antigenic response to a single immunizing dose remain poor antibody producers even on prolonged immunization.

The Immunization of Mice with Soluble Extracts of R Pneumococci.—The possibility of rendering mice actively immune to virulent pneu-

TABLE IV

The Protective Action in Mice of the Sera of Rabbits Immunized with One Single Injection of Soluble Antigen Prepared from R Pneumococci

Rabbit No.	Amount of serum	Infecting dose of Type I Pneumococcus							
		10^{-4} cc.		10^{-5} cc.		10^{-6} cc.		10^{-7} cc.	10^{-8} cc.
	cc.								
1	0.5	S	S	S	S	S	S		
2	"	D	D	D	D	D	S		
3	"	D	D	D	D	D	D		
4	"	D	D	D	D	D	D		
5	"	D	D	D	D	D	D		
6	"	D	D	D	D	D	S		
7	"	D	D	D	S	S	S		
8	"	D	S	D	S	D	S		
9	"	D	D	D	D	D	D		
10	"	D	D	D	D	D	D		
Control mice						D		D	D

S = survival of the animal.
D = death of the animal.

TABLE V

Active Immunization of Mice with a Soluble Antigen Prepared from R Pneumococci

Amount of culture Type I pneumococci	Route of immunization						Control mice
	Intraperitoneal		Subcutaneous		Intramuscular		
cc.							
10^{-4}	D 25	D 21	D 42	S	D 42	D 72	
10^{-5}	D 24	S	D 120	S	D 72	S	
10^{-6}	D 48	S	D 44	D 48	S	S	D 42
10^{-7}							D 72
10^{-8}							D 120

S = survival of the animal.
D = death of the animal; the numeral indicates the number of hours before death.

mococci, by the injection of a soluble antigen prepared from R pneumococci, is considered in Experiment 5.

Experiment 5.—The soluble antigen was prepared from the cells of 1,500 cc. of a 4 hour culture of an R Pneumococcus (derived from Type II) by the method described in Experiment 1. The filtered solution was precipitated with acetic acid at pH 4.2 and the precipitate put back in solution in 4 cc. of saline (at pH 7.0). The antigen was injected in a single dose of 0.2 cc. amounts into 18 mice by the intraperitoneal, the subcutaneous, or the intramuscular route (3 groups of 6 mice each). The animals were infected with virulent Type I pneumococci one week later.

The results of Experiment 5 indicate that some of the mice immunized with a soluble antigen prepared from R pneumococci are protected against many fatal doses of virulent Type I pneumococci. The protection, however, is of a very irregular character, and it has not been found possible so far to obtain more consistent results by varying the amount of antigen, or by increasing the number of immunizing injections.

DISCUSSION

As repeatedly shown in previous articles, it is possible to change pneumococci from the Gram-positive to the Gram-negative state without causing a disintegration or lysis of the cell bodies (1–3). This reaction is common to all types of pneumococci and to the R variants as well as the S. In the course of this limited form of autolysis, small amounts of soluble material are released in solution, and in particular a fraction which can be precipitated with acetic acid. It is this particular fraction which has been used for the immunization of rabbits and mice. This soluble antigen, whether obtained from R or S pneumococci, gives rise to the production in rabbits of antibodies which protect mice against many fatal doses of virulent pneumococci of Type I, Type II, and Type III. No effort has been made so far to achieve high protective titers in the immunized rabbits, but rather it has been attempted to establish a rapid test for comparing the antigenic efficacy of different preparations. Although there exist wide differences between individual rabbits, it was found that many animals give a positive response to the injection of a single dose of antigen. Eight antigen preparations have been tested so far; four were prepared from encapsulated Type I pneumococci, and four from an R culture (derived from Type II). All were found capable of

stimulating the production of protective antibodies in rabbits; as far as available methods permit to judge, the preparations obtained from R cells are as effective as those obtained from encapsulated cells. In all cases, the rabbit sera, when tested in mice, were found to protect much better against infection with pneumococci of Type I and Type II than of Type III; it must be pointed out, however, that the strain of Type III Pneumococcus used in these experiments is extremely virulent for the mouse, and that even Type III antisera with a high titer of anticapsular antibodies afford only little protection to mice infected with this strain.

Tillett showed that a broad immunity against infection with virulent pneumococci (Types I, II, and III) can be induced in rabbits by vaccination with avirulent R strains of pneumococcus (4). This form of active resistance is effective in the absence of demonstrable type specific antibodies; it can be passively transferred to normal rabbits by the blood of the immunized animals, but could not be transferred to mice. It is possible that the non-type specific immunity recognized by Tillett may be due to the antigen described in the present study. In the preceding experiments, the soluble antigen was used in amounts corresponding to 2 to 3 liters of broth culture per rabbit; it is likely that the amount of antigen used by Tillett in the form of intact bacterial cells (5 to 25 cc. of culture) was too small to allow the production of protective antibodies detectable by passive protection tests in mice.

Day (5, 6) and Harley (7) discovered that when virulent pneumococci are allowed to autolyze under certain conditions, an antigen is obtained which produces in mice active immunity to both homologous and heterologous types of pneumococci; the sera of rabbits immunized with this same antigen also afford non-type specific protection to mice. Day and Harley designate the antigen as "pneumococcal species antigen" and believe that it is produced by the action of the pneumococcus enzymes on the type specific antigen. Although they state definitely that this species antigen is entirely absent from the avirulent R cells, it cannot be ruled out as yet that the antigen studied in the present paper is the same as that described by the English authors.

Felton (8) has also reported that it is possible to extract from virulent pneumococci a fraction which produces specific immunity in mice, and heterologous immunity in human beings.

It is, therefore, possible to produce non-type specific immunity to pneumococcus infections by widely different techniques. Whether a single antigenic substance, the so called species antigen, is involved in all cases, or whether species specific immunity can be determined by a number of different, unrelated antigens, must remain for the present an open question.

SUMMARY

Pneumococci killed by acetic acid at pH 4.2, then allowed to become Gram-negative at pH 7.0, under conditions such that no cellular disintegration takes place, release in solution small amounts of a substance which is precipitable by acetic acid, and soluble at neutral reaction.

This soluble fraction injected into rabbits by the intravenous route causes the production of antibodies which afford definite protection to mice infected with virulent pneumococci of Types I, II, and III. Other types were not tried.

White mice immunized with this soluble antigen exhibit some active immunity to virulent pneumococci, but the results have been very irregular so far.

Soluble fractions, similar in properties and with apparently the same immunizing action, have been obtained from both virulent (S) and avirulent (R) cells of pneumococci.

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STUDIES ON EXPERIMENTAL HYPERTENSION

VII. The Production of the Malignant Phase of Hypertension*

By HARRY GOLDBLATT, M.D.

(*From the Institute of Pathology, Western Reserve University, Cleveland*)

Plate 36

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The experimental production of hypertension, accompanied or followed by the development of renal insufficiency and diffuse arteriolar hyalinization and necrosis (pale hypertension, one type, of Volhard (1), malignant hypertension of Keith, Wagener and Kernohan (2), malignant phase of essential hypertension of Fishberg (3)), has not hitherto been reported.

Methods

In a previous paper (4), the production of persistent hypertension in dogs by the constriction of the main renal arteries was published. This has also been accomplished in the monkey by the same method (5). In these and other studies, some of which have been reported (6–9), it has been found that when one renal artery is constricted adequately, the blood pressure rises in most animals and remains elevated for a variable period which lasts from weeks to months, but eventually returns to a lower, or even the original, level. In order to make the hypertension persist for years, it is necessary either to clamp the main artery of the other kidney or to remove the normal kidney. The removal of the normal kidney can be performed either at the time of the clamping of the main artery of the other kidney, or, more commonly, after the temporary hypertension due to constriction of one main renal artery has become established. The excision of the normal kidney, even after the temporary hypertension due to constriction of one main renal artery has subsided, results in a re-elevation of the blood pressure, which usually persists at a higher level than during the previous period. In most of the animals, when constriction of the main renal artery of both kidneys,

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or of the only kidney, is moderate, there is no consequent disturbance of renal function detectable by the usual studies of urine and blood, including urea and creatinine clearances, and the blood pressure remains elevated at various levels. In some of these animals the hypertension has persisted at a very high level for more than five years.

In the first paper (4) it was stated that when both main renal arteries were constricted severely at the same time, or with only a short interval between the two clampings, hypertension followed which was accompanied by severe disturbance of renal function. These animals developed typical clinical uremia which proved fatal after a variable period. Special mention was made in the first report (4)[1] of dogs 2-5 and 8-7, both of which developed severe hypertension and died due to renal insufficiency. In both dogs, at autopsy, petechiae were observed, mainly in the gastrointestinal tract, and urinary bladder.

The same gross lesions had been observed in several animals, before Nos. 2-5 and 8-7, during the period when the development of the clamp for the constriction of the renal artery was begun, but their nature was not recognized. No special significance was attached to these lesions, which were considered coincidental and probably infectious in origin, and they had not been examined microscopically at the time of the first report (4). Dog 8-7 had been dead about 36 hours in warm weather before it was examined. The tissue was useless for microscopic study. Subsequent study of the tissues of dog 2-5 revealed the nature of the lesion, but it was decided to delay making a report until a sufficient number of animals of this kind had been observed and studied to warrant conclusions about the exact nature and pathogenesis of the condition. Because it was soon found that severe constriction of both renal arteries proved rapidly fatal, and because the main purpose of the first investigation was to produce and study the pathogenesis of persistent hypertension, severe constriction of the main renal arteries of both kidneys was avoided. An interval of several weeks to months was allowed between the constriction of the two main renal arteries and it was frequently made moderate at first and later increased.

During the period from 1934–1936, the hemorrhagic lesions were again observed in three dogs. In dogs 1-33 and 2-37, the initial constriction of both main renal arteries had been made severe, with a short interval between the two clampings, as in dog 2-5. In dog 2-50, one main renal artery was first severely clamped, and, when the temporary hypertension was at its height, the ischemic kidney was removed. This was followed by prompt return of the blood pressure to the original level. After about 2 months of normal pressure, the main artery of the remaining kidney was severely constricted. The blood pressure became re-elevated and progressed rapidly to a very high level. Terminally, the mean blood pressure in the femoral artery, by the direct method, was more than 300 mm. Hg.

[1] Goldblatt *et al.* (4), Text-figs. 5 and 6.

During the last 5 days of life, intraocular hemorrhage and bleeding from the anus developed and the animal was in uremia when it was killed. The chart of the blood pressure of this dog has already been published (8).[2] At autopsy, this animal had petechiae and larger hemorrhages in the esophagus, gastro-intestinal tract, pancreas, diaphragmatic muscle and myocardium. The posterior chamber of both eyes was filled with blood.

When it was realized, as a result of the microscopic examination of the tissues of dogs 2-5, 1-33, 2-37 and 2-50, that the petechiae and larger hemorrhages were accompanied by the presence of severely degenerated, hyalinized and necrotic arterioles and that the extravasations of blood were probably due to rupture or destruction of these vessels, a deliberate attempt was made to produce the lesions by repeating the procedures used in dogs 2-5 and 2-50. It was thought at that time that the effect on the arterioles in both of these dogs might have been due to the previous sensitization of the arteriolar system to some substance circulating in the blood, as a result of the initial ischemia of one kidney, which was common to both animals.

During the past 2 years (1936-1938), the same clinical manifestations and gross lesions in the internal organs have been produced by various methods, in twelve more dogs. Common to all the methods have been hypertension and renal insufficiency due to severe reduction of the blood supply to the kidneys. In these dogs the experimental history varied. (For details, see the Summary of Experimental History at the end of this paper.) When the same procedures as in dog 2-5 were carried out on dog 3-35, there resulted the development of the same clinical manifestations and pathological lesions. When the same procedures as in dog 2-50 were carried out on dogs 2-67, 2-68, 3-05 and 3-39, all but 2-68 developed severe hypertension, renal insufficiency and the hemorrhagic lesions in various organs. Persistent hypertension and temporary moderate azotemia also developed in dog 2-68, but the renal insufficiency did not persist and the clinical signs of hemorrhages and pathological lesions of the arterioles (intraocular hemorrhage or bleeding from the intestine) did not develop. This animal is still living.

It was realized subsequently that sensitization of the arterioles is not a necessary condition for the development of the arteriolar lesions and hemorrhages, because they were observed in animals with hypertension and renal insufficiency due to the following procedures: (*a*) severe constriction of the main renal artery of one kidney followed, after an interval, by the removal of the normal kidney (No. 3-07); (*b*) unilateral nephrectomy followed by severe constriction of the main renal artery of the remaining kidney (Nos. 3-12, 3-16); (*c*) severe constriction of both main renal arteries at the same time (No. 3-41), and (*d*) severe constriction

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[2] Goldblatt (8), Fig. 3.

of the aorta immediately above the origin of the renal arteries in animals with one or both kidneys (Nos. 3-37, 3-59, 3-62, 3-63 and 3-65).[3]

The effects of the various procedures are illustrated by the individual histories given farther on.

Clinical Manifestations

In all the animals, during the terminal period, chemical examination of the blood revealed a great increase of urea, non-protein nitrogen and creatinine. Urea and creatinine clearance tests indicated great reduction of renal function. Some of the animals were anuric for several days before death. In all, the blood pressure was usually at a high level during this period, except a few hours before death, when it sometimes fell to a lower level. In most of the animals, the first clinical sign of the development of the malignant phase of the hypertension was bleeding from the anus. In two animals, the first sign of the onset of the condition was blindness, due to bilateral intra-ocular hemorrhage, and this was followed by the appearance of bleeding from the anus.

Gross Examination of the Tissues

In the gross, the only recognizable lesions were petechiae, some confluent, and some larger extravasations of blood. The distribution and severity of the lesions varied in different animals. In all, the gastrointestinal tract was affected in varying degrees. The petechiae appeared in all coats but were most prominent in mucosa and serosa. Next most common was the involvement of the pancreas and urinary bladder. Petechiae, and some larger extravasations of blood occurred in gall bladder, esophagus, brain, abdominal lymph nodes, heart (myocardium and epicardium), diaphragmatic muscle, eyes, suprarenals and gonads of some of the animals. In several of the dogs most of the organs mentioned showed these gross lesions. The organs in which no macroscopic hemorrhages were ever observed were the kidneys (except when associated with massive or localized infarction), skeletal muscles, skin, lungs, liver, thyroid and pituitary but, in some of these, hemorrhages were found on microscopic examination of the tissues.

[3] A preliminary report (10) of the production of prolonged hypertension by clamping the aorta above both renal arteries was made before the Central Society for Clinical Research, in Chicago, on November 5, 1937.

Microscopic Examination of the Tissues

In all the organs which showed macroscopic hemorrhages, and also in some of the organs in which they were not usually observed, in the gross, the microscopic pathologic changes consisted of degenerative disease of varying severity (hyalinization and necrosis) of the arterioles and fresh extravasations of blood into the tissue.

The most severe and most widespread lesions were found in all coats of the alimentary tract, with progressing severity from esophagus to rectum. The most severe lesions of arterioles were found in mucosa and suhmucosa. Pancreas, gall hladder, urinary hladder and spleen frequently showed severe hyalinization, with or without ohvious necrosis, of the arterioles. The distinction hetween severe hyalinization and necrosis was often not very sharp. In the skeletal muscles, with the exception of the diaphragm, changes were not common and consisted of relatively slight hyalinization of the intima and thickening of the media in those arteries and arterioles that had been subjected to a long period of henign hypertension preceding the onset of the malignant phase. Actual necrosis of arterioles was not observed in the arterioles of skeletal muscles. In the lungs, the only vessels that showed the changes in some of the animals were the bronchial arterioles. The pulmonary arteries were not affected. The only other organs which did not show necrosis of arterioles were the kidneys (see pathogenesis).

The source of the petechiae and larger hemorrhages was not always clearly demonstrahle. In many instances, they were ohviously due to dissecting hemorrhage through the wall or to actual rupture of the necrotic arterioles. In or near some of the hemorrhages, no necrotic arterioles were found so that they appeared to he capillary in origin. The degenerative changes in the capillaries were not clearly demonstrahle, hut small hyalinized and necrotic masses were ohserved within many of these foci of hemorrhage which might represent degenerated capillaries. See Fig. 5.

The arteriolar degenerative lesions varied in nature and severity[4] (see Figs. 1–5). In some of the arterioles, there was some hyalin immediately heneath the endothelial lining of the intima which remained intact (Fig. 1). This hyalin stained yellow in Van Gieson preparations and was situated internal to the elastic lamina. In the smallest arterioles, in which the endothelium composed the entire intima, this accumulation of hyalin constituted a suhintimal deposit. In some of the arterioles, the accumulation of hyalin was excentric, in others concentric, with partial (Fig. 2) or complete (Fig. 3) ohliteration of the lumen. The lumen of some of the vessels remained normal while part of the entire thickness of the wall was necrotic (Fig. 4). In some entirely necrotic arterioles there

[4] Illustrations of these lesions were exhibited hefore the American Medical Association, Atlantic City, June 7–11, 1937, and at the Graduate Fortnight of the New York Academy of Medicine, New York, Novemher 1–12, 1937.

was neither thickening of the wall nor reduction in the diameter of the lumen. Serial sections showed that different portions of the same arterioles could be relatively normal, partly or completely hyalinized, and partly or completely necrotic (Fig. 5), as Moritz and Oldt (11) have shown for human arteriolar disease. In larger arterioles, or small arteries, the lesion was mainly intimal, but, in some instances, even in the vessels of this size, the inner portion of the media was also affected. In the necrotic vessels, and even in some of the severely hyalinized arterioles, the elastica had defects or stained poorly but showed no reduplication (Weigert's method). Within the wall of some of the hyalinized or necrotic arterioles, red blood cells were present and some of these dissecting hemorrhages communicated directly with the lumen of the vessel and the extravascular blood. In no case was a deposit of blood pigment found, which would indicate repeated or old extravasations of blood. In all the tissues the hemorrhages were recent and indicated a terminal event. In none of the animals was there any intimal proliferation or reduplication of elastica in the arterioles and in none was there any indication that proliferation preceded hyalinization of the intima. In only one of the animals was there perivascular infiltration of polymorphonuclear leucocytes and lymphocytes around the necrotic arterioles of the intestine. This inflammation was probably due to coincidental infection.

Pathogenesis of the Arteriolar Lesions

Nothing is known about the pathogenesis of the arteriolar degeneration and necrosis which are found in many internal organs, but most frequently in the kidneys (3) and gastrointestinal tract (11), in human benign or malignant hypertension. The degenerative and necrotizing arteriolar lesions of the animals which have been described above are not distinguishable from those found in most cases of malignant hypertension in man (11) except that they are more severe and more widespread than in the latter. This indicates a greater susceptibility of the dog's arterioles to these changes. In human malignant hypertension, skeletal muscles and lungs also rarely show necrosis of arterioles, although hyalinization and other changes may occur in the muscles (12). The only striking difference between the lesions in man and dog is that in the latter the kidneys did not, while in the former they do very frequently show arteriolar necrosis. This discrepancy is easily explained and actually affords a clue to the pathogenesis of this lesion. In the animals, the intravascular pressure, within the kidney, is probably low, because the method involves the constriction of the main renal artery. In man, the intrarenal vascular tension is undoubtedly high, because there is sclerosis and

constriction of the preglomerular arterioles. In some of the larger vessels of the human kidney the lumen is also frequently narrowed, due to proliferation of the intima, but it has never been shown that the arterioles belonging to such vessels become necrotic. It may be that only those arterioles become necrotic that are subjected to the higb bursting tension as well as to the hypothetical toxic substance or substances in the blood which result from the renal insufficiency. There are some human cases in which necrosis of small renal arterioles is not found. These may be cases in which the renal insufficiency is due to widespread intimal proliferation in the small arteries and large arterioles and not to the reduction in the caliber of the preglomerular arterioles. This may also account for the difference and point to one of the probable factors and necessary conditions in the pathogenesis of arteriolar necrosis and hemorrhage, namely, elevated pressure within these vessels. That the accumulation of chemicals in the blood is not by itself a sufficient condition for the production of the arteriolar lesions, is shown by the fact that bilaterally nephrectomized dogs that develop azotemia but no hypertension (5), do not develop the generalized hyalinization and necrosis of arterioles and associated hemorrhages in the organs. That hypertension alone is not sufficient to determine the formation of the necrotizing lesions of the arterioles is shown by the fact that animals that have had severe hypertension for more than 5 years, without accompanying significant disturbance of renal function, have not developed this lesion. That the lesions of the arterioles are not due to ischemia is shown by the absence of the lesions from the severely ischemic kidneys of the dogs and their presence in organs in which there is no preexistent ischemia. In the dogs, at least, the combination of hypertension and severe disturbance of renal function, with consequent accumulation of chemical substances in the blood, are at least two of the necessary conditions for the manifestation of the arteriolar necrosis and associated hemorrhages in various organs. Since the hypertension is not present within the intrarenal blood vessels of the animals with the main renal arteries or the aorta above the origin of the renal arteries constricted, the lesion does not manifest itself there. The same explanation (absence of local hypertension) probably applies to the absence of the lesion in the pulmonary arteries of man as well

as of animals. What the nature of the chemical substance or substances is that plays a part in the production of these lesions, is not elucidated by these investigations on experimental hypertension that have been carried out so far but they do show that hypertension, severe disturbance of renal function, and generalized degenerative changes, including severe hyalinization and necrosis, of the arterioles, all indistinguishable from those found in the malignant phase of hypertension in man, can be induced experimentally by pronounced reduction of the blood supply to the kidneys.

All of the investigations that have been carried out so far (6, 8, 9 and 13–31), on the pathogenesis of the type of experimental hypertension that is produced by constriction of the main renal arteries, whether or not accompanied by renal insufficiency, indicate that a humoral mechanism, in some way of renal origin, is the primary cause. Whether the "hypothetical effect substance" (8) has a pressor effect, by direct peripheral vasoconstriction, or acts indirectly, in conjunction with some other substance, perhaps a known hormone, or whether it produces the hypertension by neutralizing a hypothetical depressor substance is not known. Whether it is the same or a different substance that is responsible for the production of the necrotic arterioles is also not known. The disturbance of the circulation which results from constriction of the main renal artery, consists, in all likelihood, of reduction of the intravascular pressure in the entire renal arterial system and of intracapillary pressure within the glomeruli, and at least initial reduction of blood flow through these functioning components of the kidney. It is of great significance that the most severe lesions observed have been in animals that have had a fairly long period of hypertension before the period of renal insufficiency occurred. In these animals, the arterioles also showed a varying amount of thickening of the media due to thickening of the muscular layer. In the animals, the necrotizing lesion in organs other than the kidneys is therefore secondary to the renal insufficiency and the hypertension, and probably represents merely an acceleration of a degenerative process at least partly initiated by the hypertension.

The pathological changes in the blood vessels of animals that have had hypertension for years, without significant disturbance of renal

function, will be reported upon at a later date, when a sufficient number of animals will have been studied to warrant conclusions about the pathogenetic relationship between hypertension alone and any pathologic changes that may be found in blood vessels, especially the arterioles. The thickening of the media already observed in the arterioles of some of the animals in the malignant phase of hypertension has been observed in some of the animals whose tissues have been examined after a long period of benign hypertension and was probably due to the preexisting phase of benign hypertension. Pathological changes in the retinal vessels of animals with persistent hypertension have already been reported (7). The animal (3-8) that showed the most severe change (obliterative hyalinization of the intima) was one that had had hypertension for more than 4 years, with accompanying moderate impairment of renal function. A complete report of the pathological changes in the eyes of dogs with benign or malignant hypertension will be made later.

SUMMARY AND CONCLUSIONS

The production of the acute malignant phase of experimental hypertension has been accomplished in seventeen dogs.

The method used to produce this type of hypertension was the same as for the benign type (4), namely, constriction of the main renal arteries, or the equivalent, constriction of the aorta above the origin of both main renal arteries, but the constriction was especially severe.

The malignant phase in the animals was characterized by hypertension, terminal renal insufficiency and the development of petechiae and larger hemorrhages in many internal organs, especially the alimentary tract. These were due to dissecting hemorrhage through, or rupture of, the wall of severely hyalinized or necrotic arterioles, or rupture of capillaries. Hyalinization and necrosis of arterioles were more severe and more widespread in animals that had a period of benign hypertension before the onset of renal insufficiency. In animals with a previous long period of benign hypertension, thickening of the media also occurred in arterioles, with or without hyalinized intima.

Elevation of blood pressure (mechanical factor) and renal insufficiency (humoral factor) are at least two of the necessary conditions for the development of the necrotic arterioles and hemorrhages. Necrotic arterioles and hemorrhages have not yet been observed in animals that have had very high blood pressure for years without renal insufficiency, nor in animals with azotemia, due to removal of both kidneys, but without hypertension. Hyalinized retinal arterioles have been observed in dogs with persistent hypertension and with moderate or no disturbance of renal function. That ischemia is not the cause of the necrosis of the arterioles is shown by their absence from the ischemic kidneys of the dogs and their widespread presence in other organs that were not ischemic.

These experiments show that the necrotic arterioles and hemorrhages are secondary to and not the primary cause of the malignant phase of hypertension.

Summary of Experimental History of the Animals

(1927–1930).—This was the period of development of the clamp for the constriction of the renal artery. During this time, petechiae and larger hemorrhages were observed in the gastrointestinal tract and some other organs of several animals in which both renal arteries had been severely constricted by clamps of various kinds. The gross hemorrhagic lesions were interpreted at that time as of infectious origin and were not subjected to microscopic examination. In these animals no determinations of blood pressure or of chemical changes in the blood had been made. The only determinations of blood pressure that were being made during this period were on normal animals, to study the natural variations of pressure during a long period. Two of these animals are still living and have had hypertension for nearly 5 years, after having been observed during a normal period of 5 years.

(1930–1934).—During this period, the gross hemorrhagic lesions were observed in various organs, but mainly in the gastrointestinal tract, of three dogs. The results of early experiments were reported in 1932 (32).

No. 2-5. Short Haired, Female, Mongrel, 16 Kg.—Feb. 1, 1932. Normal period begun. During this period, the average systolic blood pressure[5] was 146 mm. Hg.

Apr. 2, 1932. Right main renal artery severely clamped. During the following 17 days the systolic blood pressure rose to a maximum of 180 mm. Hg.

[5] In this publication, systolic blood pressure means blood pressure determined by the van Leersum carotid loop method.

Apr. 19, 1932. Left main renal artery severely clamped. Systolic blood pressure became progressively more elevated to a final level of 276 mm. Hg on the day of death. May 9, 1932, 3 days before death, became clinically uremic. Blood urea nitrogen 101 mg., non-protein nitrogen 135 mg., creatinine 6.5 mg. There was bleeding from the anus.

May 12, 1932. Died of renal insufficiency. At autopsy, there were petechiae and larger hemorrhages in the mucosa of the stomach, small and large intestine. Microscopically, in addition to the hemorrhages, there were severely hyalinized and necrotic arterioles in the mucosa, submucosa and muscularis of stomach and intestine, and some hyalinized arterioles in the pancreas.

No. 8-7. Mongrel, Female, 14 Kg.—Jan. 7, 1933. Normal period begun. Average systolic blood pressure during this period was 153 mm. Hg.

Apr. 12, 1933. Right main renal artery severely constricted (almost complete). During this period the systolic blood pressure reached 200 mm. Hg, then subsided to 170 mm. Hg.

Apr. 25, 1933. Left main renal artery severely constricted. The animal survived only 4 days. During this period the blood pressure rose progressively until it reached a systolic pressure of 300 mm. Hg on the day before death. During the last 2 days the animal was anuric and clinically uremic. B.U.N. 218 mg., N.P.N. 320 mg. There was some bleeding from the anus.

Apr. 30, 1933. Found dead. At autopsy, petechiae in the gastrointestinal tract and urinary bladder. The tissues were in poor condition, for the animal had been dead about 36 hours in a warm room. No microscopic examination of the tissues was made.

No. 1-33.—Nov. 27, 1933. Normal period begun. Average systolic blood pressure during this period was 155 mm. Hg.

Mar. 15, 1934. Left main renal artery severely constricted and a fish skin bag placed around the kidney to reduce the accessory circulation through the capsule. Maximum systolic pressure during this period was 200 mm. Hg.

Apr. 16, 1934. Right main renal artery severely constricted and the kidney encased in a fish skin bag. Blood pressure rose steadily to a systolic pressure of more than 300 mm. Hg for several days before death. On the day of death it fell to 220 mm. Hg. On that day the dog was in uremic coma, was vomiting bloody fluid and passing bloody fluid from the anus.

May 31, 1934. Killed with ether. At autopsy, there were petechiae and larger hemorrhages in the esophagus, stomach, intestine, pancreas, mesentery, myocardium and urinary bladder. Microscopically, these hemorrhages were associated with the presence of hyalinized and necrotic arterioles.

(1934–1936).—During this time the hemorrhagic lesions were observed in two more dogs following a period of hypertension and renal insufficiency.

No. 2-37. Short Haired Police, Female, 15 Kg.—Nov. 25, 1935. Normal period begun. During this period the systolic blood pressure was 174 mm. Hg.

Jan. 3, 1936. Splanchnic section and excision of lower four thoracic sympathetic ganglia on both sides. The blood pressure remained unchanged during this period; the average systolic blood pressure was 176 mm. Hg.

Jan. 21, 1936. The right main renal artery was severely constricted. The systolic blood pressure rose to a maximum of 210 mm. Hg during this period and then subsided.

Feb. 19, 1936. Systolic blood pressure 180 mm. Hg. Left main renal artery severely constricted. The systolic blood pressure rose to a maximum of 250 mm. Hg on Feb. 22, 2 days before death, when the B.U.N. was 109 mg., N.P.N. 138 mg. and Cr. 7.8 mg. On this day the animal was in clinical uremia, vomited bloody mucus and passed bloody material from the anus.

Feb. 24, 1936. The animal was found dead. At autopsy, petechiae and larger hemorrhages in the serosa and mucosa of the stomach and intestine, especially the large bowel, in the epicardium, myocardium, brain and leptomeninges. Microscopically, there were hyalinized and necrotic arterioles in the stomach and intestine. In the other organs the hemorrhages appeared to be of capillary origin.

No. 2-50. Long Haired, Female, Shepherd Collie, Young.—Apr. 4, 1936. Normal period begun. During this time the average systolic blood pressure was 150 mm. Hg and the direct blood pressure[6] was 134 mm. Hg.

May 14, 1936. The right main renal artery was severely constricted. The blood pressure rose during this period to a maximum systolic of 240 mm. Hg.

June 23, 1936. Right kidney was removed. The blood pressure fell promptly to normal, and remained down.

Sept. 3, 1936. The left main renal artery was severely constricted. The blood pressure rose again and became progressively higher until both direct and systolic pressures were more than 300 mm. Hg.

Oct. 12, 1936. B.U.N. 26.3 mg.

Oct. 14, 1936. The posterior chambers of both eyes were filled with blood. The eyeballs were tense.

Oct. 15, 1936. B.U.N. 83 mg. The animal was anuric and comatose. The direct blood pressure was 240 mm. Hg and the systolic pressure 270 mm. Hg. The dog was killed with ether. At autopsy, petechiae and larger hemorrhages were present in esophagus, stomach, small and large intestine, urinary bladder and pancreas. Microscopically, the arterioles in all these organs showed severe hyalinization and necrosis. This was most severe in the arterioles of the alimentary tract.

(1936–1938).—During this period a deliberate attempt was made to produce the arteriolar lesions by various methods, and they were also observed in other experimental animals. All of these methods had in common severe reduction of the blood supply to the kidneys.

[6] In this publication, direct blood pressure means the so called mean blood pressure in the femoral artery, determined by inserting a needle connected with a mercury manometer directly into the femoral artery.

No. 2-67. Short Haired, Mongrel Bull, Female, Middle Aged, 13.6 Kg.—May 2, 1936. Normal period begun. Average direct blood pressure 135 mm. Hg. B.U.N. 15.7 mg., N.P.N. 33 mg. and Cr. 1.4 mg. per 100 cc.

May 28, 1936. Left main renal artery severely constricted. During this period the direct blood pressure rose to a maximum of 215 mm. Hg.

Feb. 23, 1937. Direct blood pressure 200 mm. Hg. B.U.N. 14.3 mg., N.P.N. 32.1 mg., Cr. 1.6 mg. Left nephrectomy. Blood pressure fell to normal in less than 24 hours and remained down.

Mar. 31, 1937. B.U.N. 17.3 mg., N.P.N. 33.3 mg., Cr. 1.7 mg. Direct blood pressure 140 mm. Hg. The right main renal artery was severely constricted. Direct blood pressure became re-elevated to a maximum of 230 mm. Hg.

Apr. 2, 1937. B.U.N. 24.3 mg., N.P.N. 92 mg., Cr. 2.4 mg. Direct blood pressure 205 mm. Dog vomiting, looked ill and muscles were twitching. Clamp on the right main renal artery was released completely, at 4:30 p.m.

Apr. 3, 1937. 9:00 a.m. Direct blood pressure 140 mm. Hg and remained down. B.U.N. 23.3 mg., N.P.N. 37.6 mg., Cr. 1.1 mg. Twitching stopped. Animal improved rapidly.

Apr. 23, 1937. 11:00 a.m. Direct blood pressure 140 mm. Hg. Right main renal artery reconstricted, severely. Direct blood pressure again rose to maximum of 200 mm. Hg. 3:30 p.m. Direct blood pressure 175 mm. Hg. Anuria. Animal ill. Vomiting. Clamp slightly released.

May 11, 1937. Became progressively more uremic. B.U.N. 133 mg., N.P.N. 194 mg., Cr. 6.0 mg. Vomiting bloody mucus and passing blood from the anus. Killed with ether. At autopsy, petechiae and larger hemorrhages in the esophagus, stomach, and small and large intestine, pancreas, epicardium, abdominal lymph nodes, pituitary and brain. In the right optic thalamus there was one zone of hemorrhage 0.5 cm. in diameter. Microscopically, these hemorrhagic lesions were associated with the presence of hyalinized and necrotic arterioles. The lesions of the arterioles were most severe in the gastrointestinal tract, pancreas and abdominal lymph nodes.

No. 3-05. Reddish Brown Male, Mongrel Hound, Middle Aged, 17 Kg.—Dec. 1, 1936. Normal period begun. The average direct blood pressure during this period was 138 mm. Hg.

Mar. 9, 1937. Left main renal artery severely constricted. After this the direct blood pressure reached a maximum of 215 mm. Hg on Apr. 5 and May 17, 1937.

Apr. 23, 1937. Direct blood pressure 190 mm. Hg. B.U.N. 10.5 mg., N.P.N. 24.5 mg., Cr. 1.2 mg.

May 25, 1937. Left nephrectomy. The direct blood pressure fell to 140 mm. Hg, in less than 24 hours, and remained normal.

June 28, 1937. Right main renal artery severely constricted.

July 2, 1937. Direct blood pressure rose to a maximum of 200 mm. Hg on this day.

July 3, 1937. B.U.N. 147 mg., N.P.N. 181.8 mg., Cr. 6.8 mg. The animal was comatose and died while the blood pressure determination was being made. At autopsy, petechiae and larger hemorrhages in the esophagus, stomach, intestine, pancreas, myocardium, diaphragmatic muscle, mesentery, omentum, brain and retroperitoneal lymph nodes, gall bladder and urinary bladder. Microscopically, these hemorrhagic lesions were associated with the presence of hyalinized and necrotic arterioles.

No. 3-07. Female, Police, Old, 26 Kg.—Dec. 1, 1936. Normal period begun. During this time the average direct blood pressure was 145 mm. Hg.

Mar. 15, 1937. Left main renal artery severely constricted. The direct blood pressure rose during this period to a maximum of 205 mm. Hg, on Apr. 19, and then began to subside.

Apr. 22, 1937. Direct blood pressure 170 mm. Hg. B.U.N. 22.0 mg., N.P.N. 41.4 mg., Cr. 1.7 mg. Right nephrectomy. The direct blood pressure rose again to 205 mm. Hg but dropped when the animal became seriously ill.

May 10, 1937. B.U.N. 271, N.P.N. 354, Cr. 8.4. In clinical uremia. Vomiting. Bleeding from anus.

May 11, 1937. Found dead. At autopsy, petechiae in the small and large intestine. Microscopically, these petechiae were associated with partly or completely hyalinized and necrotic arterioles, but the wall of most of these vessels was not thickened and the lumen was not decreased in diameter in the larger arterioles.

No. 3-12. Female, Short Haired, Mongrel, Young, 13.5 Kg.—Jan. 5, 1937. Normal period begun. During this period the average direct blood pressure was 146 mm. Hg.

Mar. 25, 1937. Left nephrectomy and partial suprarenalectomy. Two-fifths of the cortex alone of the left suprarenal was left. During this period the average direct blood pressure was 143 mm. Hg.

Apr. 13, 1937. Right suprarenalectomy and right main renal artery severely constricted.

Apr. 23, 1937. Animal died in uremia. The direct blood pressure during this period rose to a maximum of 220 mm. Hg. The N.P.N. on the day of death was 300 mg., Cr. 12.9 mg. At autopsy, there were petechiae in the esophagus, stomach and intestine. These were most severe in the lower part of the small intestine and in the large intestine. There were also petechiae and larger hemorrhages in the epicardium and myocardium. Microscopically, these lesions were associated with hyalinized and necrotic arterioles. The necrotic arterioles were most abundant in the sections of intestine.

No. 3-16. Police, Female, Young, 14 Kg.—Feb. 9, 1937. Normal period begun. The average direct blood pressure during this period was 125 mm. Hg.

Mar. 26, 1937. Left nephrectomy and partial left suprarenalectomy. About half of the cortex alone of this suprarenal was left, the medulla of this portion was destroyed. Average direct blood pressure during this period was 121 mm. Hg.

Apr. 28, 1937. Right suprarenalectomy. Right renal artery severely clamped. During this period the direct blood pressure reached a maximum of 225 mm. Hg 2 days before death. On May 23, the day of death, it was 215 mm. Hg. N.P.N. 74 mg. and Cr. 2.6 mg. At autopsy, there was a moderate number of discrete petechiae in the small and large intestine. Microscopically, these hemorrhagic lesions, especially in the mucosa, were associated with the presence of hyalinized and necrotic arterioles.

No. 3-35. Male, Chow, Young, 12.4 Kg.—Mar. 31, 1937. Normal period begun. Average direct blood pressure 127 mm. Hg.

July 29, 1937. Left main renal artery severely constricted. Direct blood pressure rose to 185 mm. Hg and then returned eventually to 145 mm. Hg. B.U.N. 19.5 mg., Cr. 1.5 mg.

Dec. 28, 1937. Right main renal artery severely constricted.

Dec. 31, 1937. Direct blood pressure 200 mm. Hg. B.U.N. 64.5 mg., Cr. 3.6 mg.

Jan. 1, 1938. Direct blood pressure 200 mm. Hg. B.U.N. 150 mg., Cr. 6.4 mg.

Jan. 2, 1938. At 11:00 p.m., the animal was in uremic coma and at 11:30 a.m. it was found dead. At autopsy, there were petechiae and larger hemorrhages in great abundance in the esophagus, stomach, small and large intestine, pancreas, gall bladder and urinary bladder, myocardium, diaphragmatic muscle and brain. Microscopically, these hemorrhagic lesions were associated with the presence of necrotic and hyalinized arterioles. Many of the arterioles had a thickened media.

No. 3-41. Collie, Male, 13.2 Kg.—Apr. 20, 1937. Normal period begun. The average direct blood pressure during this period was 126 mm. Hg.

Oct. 9, 1937. Both main renal arteries were constricted very severely at the same time.

Oct. 11, 1937. Direct blood pressure 208 mm. Hg. B.U.N. 96 mg., Cr. 2.8 mg.

Oct. 12, 1937. Direct blood pressure 220 mm. Hg. B.U.N. 150 mg., Cr. 5.4 mg. Passing blood from urethra and anus. In uremic coma. Vomiting.

Oct. 13, 1937. Found dead. At autopsy, petechiae and hemorrhages in the stomach, small and large intestine, brain (parietal lobe). Microscopically, in the stomach and intestine, these lesions were associated with hyalinized and necrotic arterioles, without much thickening of the wall or reduction of size of lumen. In the brain, the hemorrhage appeared to be of capillary origin.

Nos. 3-37, 3-59, 3-62.—In these dogs the aorta was greatly constricted immediately above the origin of both main renal arteries.

Nos. 3-63 and 3-65.—In these dogs, the right kidney was first removed and the aorta was severely constricted above the origin of the left main renal artery. Full details about these experiments will appear in a later paper with Dr. J. R. Kahn. In these five dogs, uremia developed, and, at autopsy, there were

petechiae in the gastrointestinal tract of all and in other organs of some of the animals. Microscopically, there were hyalinized and necrotic arterioles, most abundant in the intestinal mucosa and submucosa.

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EXPLANATION OF PLATE 36

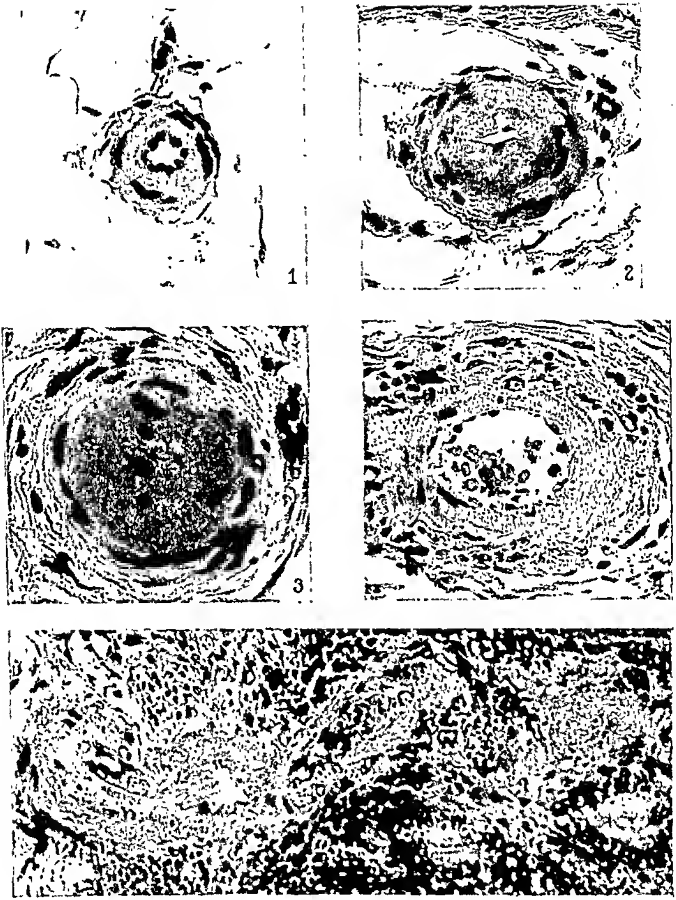
FIG. 1. Arteriole in submucosa of large intestine. Beginning subendothelial deposit of hyalin. Endothelium well preserved. Hematoxylin and eosin. ×265.

FIG. 2. Arteriole in submucosa of stomach. Obliterative hyalinization of intima, endothelium still recognizable but nuclei reduced in number and pyknotic. Hematoxylin and eosin. ×430.

FIG. 3. Arteriole in submucosa of small intestine. Lumen completely obliterated by accumulation of hyalin containing a few pyknotic nuclei. Hematoxylin and eosin. ×430.

FIG. 4. Arteriole in submucosa of stomach. Portion of entire thickness of wall necrotic. Normal thickness of wall and lumen natural size. Hematoxylin and eosin. ×325.

FIG. 5. Arteriole, cut longitudinally, in submucosa and mucosa of large intestine. Partly hyalinized, partly necrotic, with extravasated blood around it. A portion of the same arteriole, in the submucosa, immediately proximal to the part included in this figure, was entirely normal. Hematoxylin and eosin. ×255.



(Goldblatt: Hypertension. VII)

BILE AND BLOOD PLASMA CHOLESTEROL AS INFLUENCED BY BLOOD DESTRUCTION IN NORMAL AND BILE FISTULA DOGS

BY A. WRIGHT, M.D., AND W. B. HAWKINS, M.D.

(From the Department of Pathology, The University of Rochester School of Medicine and Dentistry, Rochester, New York)

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It is commonly assumed that part of the blood plasma and bile cholesterol has its origin from destroyed red cells. Search of the literature reveals no convincing data that warrant such an assumption.

Under standard conditions the normal bile fistula dog eliminates bile pigments ranging in amount from 60 to 120 mg. a day. The individual dog maintains a remarkably constant daily output. This bile pigment has two possible known sources, namely from the hemoglobin of red cells or muscle. Whipple and Robscheit-Robbins have demonstrated that dog muscle hemoglobin when given parenterally may be converted into bile pigment (12). How much bile pigment is derived from the daily disintegration of myohematin is not known. However from evidence accumulated from other studies relating to the length of the life of circulating red cells it would appear that the greater part of the bile pigment daily excreted comes from the wear and tear disintegration of the red blood cells (5).

Theoretically 1 gm. of hemoglobin is equivalent to 40 mg. of bile pigment and it has been shown that following the intravenous injection of dog hemoglobin solutions there is an increase in bile pigment elimination corresponding to 90 to 95 per cent of the expected increase if there were a quantitative transformation of the pigment radicle of hemoglobin to bile pigment (10).

Brun (2) has determined that red cells contain 140 mg. per cent of cholesterol, all of which is in the uncombined form. Since normally our dogs have a circulating hemoglobin of 130 per cent or 18 gm. (standard is 13.8 gm. per 100 cc.) then 5 cc. of blood would contain

approximately 1 gm. of hemoglobin and 4 mg. of cholesterol in the contained red cells.

If we disregard myohematin as a source of bile pigment and assume that all of this pigment comes from destroyed red cells, then at most only from 1.5 to 3 gm. of hemoglobin are being destroyed daily on the basis of 60 to 120 mg. of excreted bile pigment. This amount of hemoglobin represents from 8 to 15 cc. of red cells and with their destruction 6 to 12 mg. of cholesterol would be liberated. This amount represents a very small fraction of the total plasma cholesterol which ranges between 100 to 200 mg. per cent.

During the course of several experiments utilizing normal and bile fistula dogs, large amounts of red cells have been destroyed by means of acetyl phenylhydrazine or due to infection of the dog by *Bartonella canis* (7). Daily determinations of plasma cholesterol, bile cholesterol, and bile pigments have been made. These data indicate that *following the destruction of large numbers of red cells there is no corresponding increase in the plasma cholesterol.* There may be actually a decrease in the bile cholesterol at the height of blood destruction when much excess bile pigment is being excreted.

Methods

Two types of bile fistula dogs have been used, the sterile bag fistula as devised by Rous and McMaster (9) and the gall-bladder renal fistula of Kapsinow, Engle, and Harvey (6). In the latter fistula the common bile duct is ligated and cut and the fundus of the gall-bladder is sutured into the renal pelvis. The dogs are kept in galvanized iron metabolism cages and the urine-bile mixture is collected over 24 hour periods. Chloroform 5 cc. is used as preservative. Such fistula dogs can be maintained in excellent physical condition for long periods of time if they are fed properly and if dog or ox bile (50 to 75 cc.) is given daily. The bile is given either by stomach tube or mixed with the food and it is beneficial in preventing abnormalities resulting from total bile deprivation (4).

The dogs are fed either kennel diet or salmon bread mixture. The former consists of mixed hospital kitchen scraps, and the salmon bread, baked in the laboratory, contains wheat flour, potato starch, salmon, tomatoes, bran, and a salt mixture. Its preparation has been previously described (11). These diets are suitable for bile fistula dogs as they are rich in carbohydrates, adequate in protein, but low in fat.

Methods of hemoglobin, blood volume, and bile pigment determination have been described elsewhere (11, 7).

The dogs are bled every morning at the same time and fed in the early afternoon

so the blood samples are free from the questionable influence of alimentary absorption. Approximately 10 cc. of blood drawn from the jugular vein are received into a 15 cc. calibrated hematocrit tube containing 2 cc. of a solution of 1.4 per cent sodium oxalate and immediately centrifugalized for 35 minutes.

Total plasma cholesterol has been determined following the method of Bloor (1) with slight modifications. The method for determining the cholesterol esters is given in detail since changes have been made in the method formerly used. The addition of ethyl ether as an extractive along with petroleum ether insures uniform and complete extraction of the cholesterol esters, whereas when petroleum ether alone is used, the values obtained are lower and tend to vary. The ethyl ether should be fresh and of purest anesthesia grade.

Method for the Colorimetric Determination of Blood Plasma Cholesterol.—Pipette 5 cc. of plasma into 75 cc. of alcohol-ether extraction mixture (one part ethyl ether to 3 parts of 95 per cent alcohol) in a 100 cc. volumetric flask. The plasma is run slowly into the extraction mixture as the flask is actively shaken; a fine white curd-like precipitate forms. The mixture is brought carefully to the boiling point (steam bath) for 30 seconds, stoppered, cooled to room temperature, and the volume made up to 100 cc. with the alcohol-ether mixture. The mixture is then filtered; the filtrate is placed in a tightly stoppered flask. The precipitate is discarded.

Determination of Total Cholesterol.—An aliquot[1] of the alcohol-ether extract is placed in a 125 cc. flask, a small stirring rod inserted, and evaporated to dryness on the steam bath. To the dry residue in the flask, 5 cc. of $CHCl_3$ are added, boiled down to about 2 cc. volume, and poured into a 10 cc. glass stoppered, graduated cylinder. This is repeated twice more. The final volume of the combined $CHCl_3$ extracts should not exceed 7 cc.

Color Development.—To the $CHCl_3$ extract in the graduated cylinder, add 2 cc. of acetic anhydride and 0.1 cc. concentrate H_2SO_4; stopper, mix gently, and make up to 10 cc. with $CHCl_3$. The color development is made at room temperature which should not vary appreciably from 25°C. The colorimetric readings are made 20 minutes after addition of the H_2SO_4. The color is maintained at its maximum for about 15 minutes, during which time colorimetry should be completed.

Determination of Esterified Cholesterol.—To an aliquot[1] of the extract in a 125 cc. flask, add 2 cc. of 0.5 per cent digitonin in 95 per cent alcohol, insert a glass stirring rod, and evaporate to dryness. To the residue in the flask add 25 cc. of petroleum ether (redistilled fraction boiling below 60°C.), boil this down to about 10 to 12 cc., and pour through a sintered glass filter under gentle suction into a

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[1] *Aliquots.*—The amount of extract taken for an aliquot should closely approximate in cholesterol content the amount of cholesterol used in the standard. For most normal plasmas an aliquot of 10 cc. of extract for the total cholesterol and 15 cc. for esterified cholesterol is satisfactory when 5 cc. of plasma are used.

125 cc. flask. This is repeated 3 times more, using 25 cc. of ethyl ether instead of petroleum ether. (The sintered glass filter is of the type marked 4 G 4, Schott and Gen, Jena). The combined volume of the one petroleum ether and the 3 ethyl ether extracts is evaporated to dryness on the steam bath. The residue in the flask is extracted with $CHCl_3$ and the color developed in the method for total cholesterol.

Determination of Bile Cholesterol.—The method of Elman and Taussig (3) was used for the determination of cholesterol in bile with development of the color reaction as detailed in the method of blood plasma cholesterol.

EXPERIMENTAL OBSERVATIONS

Table 1 (dog 35-17) illustrates the results of a period of blood destruction with acetyl phenylhydrazine in a normal dog. This dog, an adult female mongrel, weight 16.8 kg., was fed the basal salmon bread diet beginning 10 days before control observations were made. After 7 days of observation for a control period, the dog was given subcutaneously on 2 consecutive days, 100 mg. of acetyl phenylhydrazine dissolved in normal saline. After this the red cell percentage began to decrease, there was marked bilirubinemia, and the urine was deeply pigmented. 3 days later the same amount of phenylhydrazine was given and then on alternate days for five doses. Through this period the hemoglobin decreased rapidly from a level of 136 per cent to 58 per cent. A slight increase in the blood plasma cholesterol was noted during the first 5 days of blood destruction. After the injections of drug were stopped, the bilirubinemia cleared rapidly and the hemoglobin increased to 103 per cent in 9 days. During the recovery period from the acute anemia there were no significant changes in the plasma cholesterol values. This experiment was repeated twice on each of two other normal dogs with similar results. Each one of these dogs lost during periods of acute blood destruction about one-half of its circulating red cells without appreciable change in the blood plasma cholesterol values.

Table 2 (dog 34-212) illustrates the results of a similar period of blood destruction in a bag fistula dog. The dog, an adult female hound, weight 17 kg., was fed a diet of kitchen scraps to which was added daily 100 cc. of ox bile. The dog maintained its weight and consumed all of its food through the experiment. After a 7 day control period, the dog was given acetyl phenylhydrazine subcutaneously

in 6 doses of 100 mg. each as indicated in the table. The excretion of bile pigment increased rapidly with continued administration of the drug, and the hemoglobin decreased from a level of 133 per cent to 59 per cent. During this period the blood plasma cholesterol values

TABLE 1

Blood Plasma Cholesterol in Normal Dog (35-17)
Blood Destruction by Acetyl Phenylhydrazine

Date	Acetyl phenylhydrazine	Total blood plasma cholesterol	Esters blood plasma cholesterol	Esters of total	Hemoglobin
	mg.	*mg. per cent*	*mg. per cent*	*per cent*	*per cent*
Jan. 4		108	70	65	136
5		118	80	68	
6		111	73	66	
7	100	109	67	61	
9	100	123	86	70	135
10		161	109	68	
11		118	81	69	
12		121	87	72	
13	100	145	98	68	
14		119	82	69	
15	100	113	78	69	
16		117	91	78	85
17	100	104	69	66	
18		100	77	77	
19	100	108	72	67	
20	100	110	78	71	59
21		108	73	68	58
22		99	63	64	
23		104	75	72	
24		100	69	69	
25		105	71	68	
26		104	67	64	
27		121	79	65	
28		124	75	60	
29		122	74	61	
30		117	78	67	103

were slightly lower, but the percentage of esterified cholesterol remained unchanged. The bile cholesterol decreased from a level of around 30 mg. to a low point of 21 mg. on the days when the bile pigment elimination was high. Through the following period in which the hemoglobin returned to a level of 124 per cent the blood plasma

and bile cholesterol returned to the control levels. During the experiment 5360 mg. of bile pigment were excreted in excess over control amounts and this is equivalent to 134 gm. of hemoglobin destroyed (40 mg. bile pigment = 1 gm. hemoglobin). The quantity of blood

TABLE 2

Bile and Blood Plasma Cholesterol in Closed Fistula Dog (34-212)
Blood Destruction by Acetyl Phenylhydrazine

Date	Acetyl phenylhydrazine	Total blood plasma cholesterol	Esters blood plasma cholesterol	Esters of total	Bile cholesterol	Bile pigments	Hemoglobin
	mg.	*mg. per cent*	*mg. per cent*	*per cent*	*mg.*	*mg.*	*per cent*
Oct. 26		201	157	78	30	129	133
27		221	167	76	33	73	
28		190	149	78	35	100	
29	100	201	160	79	32	73	
30	100	193	151	78	30	97	
31	100	210	152	72	31	131	
Nov. 1	100	197	135	69	32	250	102
2		182	142	78	27	238	
3	100	184	144	78	30	502	
4	100	176	146	73	30	726	
5		207	148	71	24	1020	
6		180	142	79	22	1102	59
7		182	140	76	21	857	
8		185	138	75	24	519	
9		181	133	74	25	319	
10		193	135	70	29	333	
11		189	135	72	30	304	
13		214	174	81	35	235	
14		212	166	78	39	161	
15		197	128	65	37	144	
16		195	145	74	34	89	
17		210	164	78	35	74	
18		211	155	74	33	95	124

represented by this amount of hemoglobin would contain approximately 536 mg. of cholesterol.

A similar experiment was repeated once again on this same dog and once on another bag fistula dog with comparable results.

Table 3 (dog 34-211) gives the data obtained from a renal fistula dog that weighed 18.5 kg. and was being fed the salmon bread diet plus ox

bile 50 cc. and dog bile 50 cc. daily. Food consumption was 100 per cent and the weight was maintained. After a 7 day control period the dog was given acetyl phenylhydrazine subcutaneously in ten doses of 100 mg. each over a period of 14 days. The bile pigment excreted increased rapidly and with continued administration of the drug the

TABLE 3

Blood Plasma Cholesterol in Renal Fistula Dog (34-211)
Blood Destruction by Acetyl Phenylhydrazine

Date	Acetyl phenylhydrazine	Total blood plasma cholesterol	Esters blood plasma cholesterol	Esters of total	Bile pigments	Hemoglobin
	mg.	*mg. per cent*	*mg. per cent*	*per cent*	*mg.*	*per cent*
Dec. 13		105	74	70	59	127
14		111	86	77	81	
16	100	102	67	66	66	
17	100	112	76	68	203	118
18		107	71	66	149	
19	100	96	63	66	320	
20	100	92	57	62	317	
21	100	94	60	64	338	
24	100	88	55	63	790	
27	100	83	51	62	742	60
28	100	84	59	70	637	
29	100	79	55	70	399	
30	100	71	43	61	427	
31		73	45	62	360	71
Jan. 2		83	60	72	288	
3		82	59	72	194	
5		87	62	71	172	
6		88	68	77	151	
7		91	66	73	227	99
8		83	57	69	144	
9		89	63	71	212	
10		99	73	74	120	106

hemoglobin decreased from 118 to 60 per cent and the blood plasma cholesterol levels showed a slight but definite decrease. During the following 12 days the total blood plasma cholesterol and esterified cholesterol values remained slightly lower than the control levels. The bile pigments returned to the control levels 2 weeks after the last day given in the table. This delay was due to the marked bilirubinemia resulting from the blood destruction. The bile pigment excreted

above control amounts during the experimental period of 39 days was 8829 mg. which is equivalent to 220 gm. of destroyed hemoglobin. With the destruction of this much hemoglobin approximately 880 mg. of cholesterol would have been liberated from disintegrated red cells.

This experiment was repeated twice on another gall-bladder renal fistula dog with similar results.

TABLE 4

Bile and Blood Plasma Cholesterol in Closed Fistula Dog (33-51)
Blood Destruction by Bartonella canis

Date	Total blood plasma cholesterol	Bile cholesterol	Bile pigments	Hemoglobin
	mg. per cent	*mg.*	*mg.*	*per cent*
Nov. 17	158	29	103	106
18	127	36	84	
19	128	28	73	
20	145	14	85	
21	120	11	370	
22	105	9	815	
23	95	13	600	94
24	106	15	377	
25	93	16	474	
26	109	12	444	
27	94	16	392	
28	84	16	642	79
29	80	15	660	
30	87	13	727	
Dec. 1	83	13	724	
2	87	20	342	
3	137	19	174	
4	112	23	137	
5	132	31	116	100
6	120	18	127	
7	155	20	183	

Table 4 (dog 33-51) illustrates the result of blood destruction as the result of *Bartonella canis* infection in a splenectomized dog (7) carrying a bag fistula. It was a mongrel female weighing 17.5 kg. The diet was the salmon bread mixture and it was consumed and the weight was maintained throughout the experiment. 9 days after the operation at which the fistula was established and splenectomy performed,

the dog was given whole blood (5 cc.) intravenously from another splenectomized bile fistula dog with a demonstrated *Bartonella canis* infection. As indicated in the table the bile pigment rose markedly on the 5th day after the introduction of the infected blood.

In the following period large amounts of bile pigment were excreted and this was accompanied by bilirubinemia and a decrease in the circulating hemoglobin. *Bartonella canis* bodies were demonstrated in the red cells at this time. The excess of bile pigment eliminated amounted to 5927 mg. and this is equivalent to 148 gm. of hemoglobin. As the result of destruction of red cells containing the hemoglobin one might expect 592 mg. of cholesterol to be liberated. The blood plasma cholesterol during this period was slightly lower than the control level and the bile cholesterol much lower. As the bile pigments rose to a maximum the biliary cholesterol decreased markedly; as the pigment excretion decreased, the bile cholesterol increased until both constituents reached normal levels. This inverse relationship between the bile pigments and cholesterol was noted repeatedly in this dog during later periods of blood destruction and in another splenectomized bile fistula dog also infected with *Bartonella canis.* Although a considerable amount of blood is destroyed as the result of the infection, no increase in the blood cholesterol occurred during any of the periods.

DISCUSSION

As yet there is no conclusive evidence that indicates that bile pigment is formed except from the hemoglobin of muscle and red cells that is daily undergoing disintegration. Since these dogs were kept under standard conditions throughout the experiments, any excess bile pigment eliminated above the control level may be attributed to the destruction of red cells by the acetyl phenylhydrazine or due to the *Bartonella canis.*

In these normal and bile fistula dogs large numbers of red cells have been destroyed with consequent liberation of hemoglobin in a short period of time. Basing the amount of hemoglobin destroyed on the excess bile pigment eliminated, we may state that from 134 to 220 gm. hemoglobin have been broken down in periods of from 8 to 12 days. Since 1 gm. of hemoglobin is approximately equivalent to 4 mg. of cholesterol, then from 536 to 880 mg. of cholesterol should have been

liberated during the period of blood destruction. This amount even though spread over several days should be sufficient to raise the plasma cholesterol levels. If we take 200 mg. per cent as the higher level of cholesterol normally present in the plasma, then dogs of an average weight of 17 kg. and 800 cc. plasma volume would have not more than 1600 mg. of cholesterol in the circulating plasma. However the data show that there was no increase but rather a slight decrease during the periods of red cell destruction and anemia. The percentage of esterified cholesterol remains constant. With the development of marked anemia there is some dilution of plasma and the slight decrease of total cholesterol may be the result of this dilution and therefore have no true significance.

If under the conditions of marked and quite rapid red cell destruction there is no increase in circulating plasma cholesterol, certainly the assumption that the normal daily destruction of red cells is a factor in contributing cholesterol to the plasma is not warranted.

There is no question but what cholesterol is liberated as the result of red cell destruction but it is possible that the stroma and cholesterol of the destroyed cells are taken into the phagocytic cells of the body and there gradually metabolized for the benefit of red cell production, and consequently the cholesterol is not set free in the plasma.

It is interesting to speculate as to the cause of the decreased bile cholesterol during the periods of marked pigment elimination as noted repeatedly in the dogs infected with *Bartonella canis.*

This decrease is apparent also in the bile of the dogs made anemic by acetyl phenylhydrazine. During the period of marked jaundice, the liver cells may be mildly injured and consequently the function be deranged or it is possible that the bile canaliculi may be obstructed by bile pigments which are being eliminated in such excess amounts. Autopsies on other bile fistula dogs infected with *Bartonella canis* have revealed that many of the bile canaliculi are filled with plugs of bile pigment (8).

CONCLUSIONS

Destruction of large amounts of red cells in normal and bile fistula dogs by means of acetyl phenylhydrazine causes no significant alterations of the blood plasma but there is some decrease in the bile cholesterol.

During *Bartonella canis* infection the splenectomized bile fistula dog periodically breaks down large quantities of red cells with slight decrease in the plasma cholesterol and marked decrease in the bile cholesterol.

In the periods of blood regeneration following such acute anemias there are no significant alterations in the values for plasma or bile cholesterol.

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WHITE CELL MORPHOLOGY IN RABBITS WITH INDUCED PERITONEAL EXUDATES*

By ERIC PONDER, M.D., AND JOHN MACLEOD

(From The Biological Laboratory, Cold Spring Harbor, Long Island)

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When a peritoneal exudate is induced in the rabbit by the method of Mudd, Lucké, McCutcheon, and Strumia (1), it can be shown that the number of white cells appearing in the exudate is greater than the number of such cells in the animal's circulating blood. This withdrawal of cells results in a stimulation of the bone marrow, and by inducing several exudates the rabbit's marrow can be thrown into a state of extreme stress. This paper is concerned with the morphology of the young cells which appear in the circulation, and with the morphology of the white cells contained in the exudates.

Production of Exudates

The method used for the production of the peritoneal exudates was similar to that of Mudd, Lucké, McCutcheon, and Strumia (1). About 300 cc. of sterile 1 per cent NaCl, or of Ringer's solution,[1] are injected intraperitoneally, and 18 hours afterwards the fluid is drawn off without further injection of saline. This fluid is very rich in leucocytes, usually containing approximately 0.25 cc. of cells for each 15 cc. of exudate, and in the case of a single exudate at least 90 per cent of the cells are polymorphonuclear leucocytes.

The procedure can be repeated at intervals, and we have used the same animals repeatedly for a period of several months. In order to produce extreme marrow stress, it is necessary to produce the exudates frequently, and most of the work with which this paper is concerned was carried out on a colony of twelve mature rabbits whose weights ranged from 2.5 to 4 kilos, four successive exudates being

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[1] We have injected both 1 per cent NaCl and a balanced buffered Ringer's fluid into the peritoneal cavity, without noticing any difference in the exudates which result. One would expect toxic effects to result from the injection of such large quantities of NaCl, but we have never observed such.

produced at intervals of 10 days, and thereafter more frequently, so as to bring about the death of the animal in some cases.

The blood picture in the circulation was followed by making total white cell counts, differential counts, and polynuclear counts (Cooke and Ponder, 2) at frequent intervals. Red cell counts and reticulocyte counts were made as required, and supravital counts were carried out on the cells of the exudates.

Effects of Producing Single Peritoneal Exudates

For some reason, at present obscure, the only cells which leave the circulation and appear in the peritoneal exudates induced by the injection of isotonic NaCl or Ringer's solution are the polymorphonuclear leucocytes, as can be shown by supravital staining. The sequence of events can be seen from the figures of Table I, which show the total white cell counts and percentages of polymorphonuclear leucocytes in the blood stream of a typical animal at various times after the saline injection, together with the total polymorphonuclear counts and the means of the polynuclear counts.

TABLE I

Time after injection	Total count per c.mm.	Polymorphonuclear leucocytes	Polymorphonuclear leucocytes per c.mm.	Polynuclear mean
hrs.		*per cent*		
0	7,400	36	2,660	2.06
2	8,600	50	4,300	1.53
4	3,050	59	1,800	1.14
6	3,650	61	2,200	1.10
8	5,300	68	3,600	1.13
24	11,000	32	3,460	1.22

The simplest interpretation of these figures is that shortly after the injection of the isotonic NaCl the polymorphonuclear leucocytes start to leave the blood stream to appear in the exudate. This is followed by a marrow stimulation, so that at the end of 2 hours the total number of circulating polymorphonuclear leucocytes is increased, while the polynuclear mean has fallen from 2.06 to 1.53 because of the addition of young cells of class I to the circulation. Thereafter, the picture is one of a competition between production of young cells from the marrow, as indicated by the continued fall in the polynuclear

mean, and a passage of polymorphonuclear leucocytes from the blood stream into the exudate, as indicated by the fall in the total number of circulating polymorphonuclear leucocytes. After 24 hours the greater part of the effect seems to be over, and the polynuclear mean has begun to increase because of the development of the cells of class I into cells of class II (Ponder, 3), this increase in the mean continues, so that the original figure of 2.06 is reached in less than 7 days.[2]

In experiments of this kind, the question always arises: What is the relation between the number and kind of the cells found in the exudate and the number and kind of the cells in the circulating blood? The cells of the exudate must, of course, have at one time been cells of the blood stream, the composition of which varies from the time at which the exudate begins to be produced, until the time at which it is withdrawn. Table I, however, shows clearly that the bone marrow stimu-

[2] Our experience bas been peculiar as regards the length of time required for a deflected polynuclear count to return to normal. In 1926 one of us (3) described the deflection of the polynuclear count in the rabbit after the injection of thyroid extract, and found that a complete return to the steady state took from 14 to 21 days; it was therefore concluded that this is the normal lifetime of the polymorphonuclear leucocyte in the blood stream. This result was confirmed, in its general aspects, by Ponder and Flint (4), deflection with various drugs and extracts, including nucleic acid, Kennedy and Grover (5), deflection by x-rays, Kennedy and Thompson *(6), deflection* by ultraviolet light, Charipper (7), deflection by thyroid extract in *Necturus*, Danzer (8), deflection by tissue extracts, and Climenko (9), deflection by ergosterol and ultraviolet light. In this series of experiments, we have found the polynuclear count to return to its steady state much more rapidly after a deflection. When the deflection occurs as a result of a single injection of nucleic acid (1 mg. per kilo), the count returns to the original steady state within 4 days and usually more rapidly; when a single intraperitoneal exudate is produced, the return may take about 7 days, but in this case the stimulus may be operative for a longer period. The only suggestion we can make for this more rapid return to an apparently steady state is that the polynuclear counts of all our rabbits were more left-handed than those of the animals used in the investigations mentioned above. This seems to be another case of either environmental or genetic variation in the count (Macleod, 10). The more left-handed the count, the more rapidly would one expect a return from a deflection, and there is the additional difficulty that wben the count is left-handed, there are few classes containing cells, and so it is more difficult to follow the successive maxima in the classes, wbich act as a guide to the rate of development of the polymorphonuclear leucocyte.

lation, and the pouring of young cells into the blood stream, precedes the leucopenia which presumably, if not necessarily, corresponds to the passage of the greatest number of cells into the exudate; the type of cell in the exudate accordingly approximates more nearly to the type of cell in the circulation at the time when the exudate is withdrawn than to the type of cell initially present. Supravital staining of the cells of the exudate is not altogether satisfactory; one can show that the great majority of the cells are neutrophiles, but it is impossible to do an accurate polynuclear count.

The exudate is withdrawn after 18 hours, and in this particular case, 60 cc. of fluid containing 7,000 cells per c.mm. were obtained. The amount of fluid which can be withdrawn, however, is always less than the total amount in the peritoneal cavity, and the total amount can be determined only by killing the animal. This was done in several cases, and the average amount of fluid recovered was 130 cc. with an average cell content of 6,500 per c.mm.; this means that 8.5 (10^8) cells, nearly all polymorphonuclear leucocytes, passed into the exudate from the blood stream which, on an average, contains about 3 (10^8) polymorphonuclear leucocytes, and so the number of polymorphonuclear leucocytes which pass into the exudate is greater than the entire number in the circulation. The amount of intraperitoneal saline injected, however, is really enormous, being more than one and a half times the blood volume of the rabbit.

The Mechanism of the Marrow Stimulation

Without considering why only polymorphonuclear leucocytes leave the circulation to appear in the exudate, some light can be thrown on the nature of the marrow stimulation which appears shortly after the intraperitoneal injection of isotonic NaCl. Danzer (8) has shown that subcutaneous injection of saline extracts of a variety of tissues (muscle, liver, testicle, brain, etc.) produces a marrow stimulation and a deflection of the polynuclear count similar to that which follows the injection of nucleic acid, and that the stimulating substance is associated with the protein fractions. By centrifuging a portion of the intraperitoneal exudate, a supernatant fluid can be obtained, the injection of which into another rabbit brings about a marrow stimulation and an extreme deflection of the polynuclear count; a typical experi-

ment is shown in Table II, in which 10 cc. of supernatant fluid from an exudate was injected intraperitoneally into a second rabbit.

Comparing this table with Table I, it is clear that in each case there is a marrow stimulation with a deflection of the polynuclear count, although the transient leucopenia which occurs in the case of the intraperitoneal exudate does not occur in the case of the injection of the supernatant fluid, because there is no withdrawal of neutrophiles from the blood stream into the abdominal cavity. When an intraperitoneal injection of saline is given, and the leucocytes leave the blood stream to appear in the exudate, it is hard to conceive that a mere absence of cells from the circulation should result in a stimulation of the marrow; it is much more probable that disintegration products of the cells

TABLE II

Time after injection	Total count per c.mm.	Polymorphonuclear leucocytes	Polymorphonuclear leucocytes per c.mm.	Polynuclear mean
hrs.		*per cent*		
0	9,300	27	2,500	1.58
1.5	6,900	33	2,280	1.31
3.0	8,950	53	4,750	1.12
4.0	8,050	64	5,100	1.18
6.0	7,950	67	5,350	1.30
9.0	7,850	48	3,760	1.22
22.0	10,800	26	2,800	1.41

which first appear in the exudate are reabsorbed, and that these are the stimulating agents.

The Effect of Producing Repeated Exudates

The time required for the polynuclear count to return to normal after the production of a single exudate is about 4 days, and if a second exudate is produced after this time the same sequence of events follows as in the case of the first exudate, the circulation being flooded with young cells, and the polynuclear count deflecting to the left. When the exudate is drawn off 18 hours after injection of the saline, over 90 per cent of the cells are again found to be young polymorphonuclear leucocytes. After allowing an interval for recovery, the process can be repeated again and again, but after exudates have been produced several times the picture begins to present new features.

As the exudates are repeatedly produced, the marrow responds by throwing younger and younger cells into the circulation. In a colony of 12 rabbits, we produced 4 successive exudates in each animal, with an average interval of 10 days between each exudate, and after the injection of saline for the fourth exudate, the circulating polymorphonuclear leucocytes were all very young cells of class I, or even metamyelocytes. These young cells, however, are morphologically different from the normal polymorphonuclear leucocyte; the nucleus is large and single lobed, and its chromatin content is relatively poor and irregularly distributed, so that some areas in the nucleus stain deeply while others do not stain at all. The cytoplasm contains large granules which are not all oxyphile, as in the normal rabbit polymorphonuclear leucocyte, but mixed, some being oxyphile and others azurophile. The cells have also a tendency to be larger than the normal polymorphonuclear leucocyte, their characteristics, in fact, are very similar to those of the macropolycyte (Cooke and Ponder, 2) which appears under conditions of extreme marrow stress, except that the nucleus is not hyper-segmented.[3]

In the case of some of the animals, after having obtained 4 successive exudates at intervals of 10 days, we gave several intraperitoneal injections of isotonic saline at 2 day intervals, withdrawing each exudate after 18 hours, as before. Metamyelocytes and myelocytes then appear in the circulation, until after 4 or 5 exudates in rapid succession the polymorphonuclear leucocytes of the blood stream are entirely replaced by myelocytes. Finally the animal develops a severe leucopenia (average count about 1,500 cells per c.mm.), and dies shortly thereafter.

In contrast to the production of exudates at intervals of less than 3

[3] In a recent paper Jones (11) has pointed out that the macropolycyte is not necessarily multi-lobed and has given a description of the macropolycyte in pernicious anemia which is almost identical with our description of the cells found after repeated peritoneal exudates. The same type of cell is almost invariably found after an intravenous injection of sodium nucleinate (1 mg. per kilo), and they seem to be the result of a hurried maturation in a marrow under stress. On a single occasion an intravenous injection of sodium nucleinate resulted in a flooding of the blood stream with macropolycytes having as many as 5 to 10 lobes each. We have not observed this particular phenomenon again, but it is doubtless merely another manifestation of what is essentially the same process.

days, when leucopenia and death result, if exudates are produced at intervals of from 3 to 4 days the marrow seems capable of delivering polymorphonuclear leucocytes into the circulation almost indefinitely. Its response seems, indeed to be almost of an "all or none" nature; given about 2 days rest between repeated stimulations such as we use, it will respond to each new stimulus in virtually the same way; stimulated a little more frequently, however, it rapidly fails to deliver even young polymorphonuclear leucocytes and liberates metamyelocytes and myelocytes in diminishing numbers.

Examination of the red cell counts shows that the total number remains at a comparatively constant level, even when repeated exudates are produced. We have observed, however, that considerable numbers of normoblasts, amounting to as many as 10 per cent, may appear in the circulation at the height of the marrow response to a demand for new white cells. Injection of nucleic acid may also, in some cases, be followed by the appearance of normoblasts, but, as in the case of the intraperitoneal injection of saline, the appearance of these is quite irregular, and apparently dependent on some factor which we have not been able to control.

SUMMARY

1. When peritoneal exudates are produced in the rabbit by the injection of a large volume of isotonic saline, nearly all the cells in the exudate are polymorphonuclear leucocytes, and the number contained in a single exudate may exceed the entire number originally present in the circulation.

2. The migration of polymorphonuclear leucocytes from the blood stream into the exudate is followed by a stimulation of the marrow, so that the blood stream is filled with young cells, many of which also pass into the exudate. This marrow stimulation, with the resultant shift of the polynuclear count to the left, is probably produced by the absorption of breakdown products of the cells which first appear in the exudate.

3. If exudates are produced repeatedly at intervals of from 4 to 10 days, the marrow responds by throwing younger and younger cells into the circulation, so that the blood stream becomes full of very young polymorphonuclear leucocytes of class I, or even metamyelo-

cytes. If 4 or 5 exudates are produced in rapid succession, the polymorphonuclear leucocytes in the circulation are all replaced by metamyelocytes and myelocytes.

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STUDIES ON AN UNCOMPLICATED CORYZA OF THE DOMESTIC FOWL

IX. The Cooperative Action of Hemophilus gallinarum and the Coccobacilliform Bodies in the Coryza of Rapid Onset and Long Duration

By JOHN B. NELSON, Ph.D.

(*From the Department of Animal and Plant Pathology of The Rockefeller Institute for Medical Research, Princeton, New Jersey*)

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In an earlier paper on fowl coryza it was noted that the disease produced by the intranasal injection of normal birds with exudate from the upper air passages of naturally infected fowl could be divided into three types. Type I was characterized by a rapid onset and short duration, type II by a slow onset and long duration, and type III by a rapid onset and long duration (1).

A Gram-negative bacillus corresponding to the hemophilic bacterium originally described by De Blieck was regularly isolated from birds infected with type I coryza (2,3). There is reason to believe that this bacillus, now known as *Hemophilus gallinarum*, was the only etiologic factor involved. The relation of *H. gallinarum* to the type III coryza with which it was also associated was less clearly defined. Recently isolated cultures were invariably infective but produced a coryza of short duration in contrast to the prolonged course of the coryza produced by exudate. Confusing irregularities were also observed following the injection of exudate in birds which had recovered from the coryza produced by *H. gallinarum*.

We had previously suggested that the differences between the coryzas produced by *H. gallinarum* and type III exudate might be referable to a degradation in virulence induced by artificial cultivation of the bacillus (1). This suggestion was made before the etiology of type II coryza had been worked out. With the demonstration that this type was caused by minute Gram-negative cells termed coccobacilliform bodies, another explanation was offered (4): namely, that the type III coryza might be due to a mixed infection with these

bodies and *H. gallinarum*. Observations bearing on this association in relation to the etiology of the coryza of rapid onset and long duration are here presented.

The Demonstration of Coccobacilliform Bodies in Exudate from Type III Coryza

The first evidence that coccobacilliform bodies were associated with type III coryza was obtained from birds injected with exudate after recovery from the coryza produced by *H. gallinarum* in pure culture.

Strains of types II and III have been maintained since the fall of 1933 in birds of known history by the serial passage of exudate originally secured from natural cases. In these two groups of birds which are held under strict quarantine in separate units the two types have retained their respective characteristics throughout the long series of transfers. The exudate and the cultures of the two infective agents used in the following experiments were obtained from these birds.

Five normal birds were injected intranasally by way of the palatine cleft with approximately 0.5 cc. of the fluid from the base of a 48 hour horse blood agar culture of *H. gallinarum* (the 4th subculture since isolation). The birds in this experiment and the succeeding ones were Rhode Island Reds, 8 to 12 weeks old, from a flock reared at the Institute. They were placed in single cages in the same isolation unit with the birds infected with the stock strain of type III coryza. This quarantine unit is large and has a separate compartment at one end which affords partial isolation. The injected birds were held in this compartment and examined daily. 2 to 3 weeks after the nasal discharge had subsided they were injected with 0.5 cc. of a broth suspension of type III exudate. The reaction produced by this exudate is so constant that only a single control was included. Several days after the appearance of a discharge the birds were killed and autopsied. The nasal exudate was examined culturally and microscopically and tested for infectivity by injection in normal birds.

As indicated in Table I the 5 birds injected with *H. gallinarum* showed a nasal discharge after a brief incubation period of 1 to 3 days. The duration of the nasal discharge was also short, 5 to 13 days. The injection of type III exudate after recovery from the initial coryza was followed by a second coryza which was of slow onset, 13 to 28 days. The bacteriological examination of exudate from these birds, made 1 to 5 days after the onset of the second coryza, failed to demonstrate *H. gallinarum*, but did indicate the presence of coccobacilliform bodies. The infectivity tests showed a coryza of slow onset, 16 to 23 days, and of long duration, 34 to 58 days. The single

normal bird injected with type III exudate developed a coryza on the 2nd day, *H. gallinarum* being recovered from the nasal exudate.

The inoculation of tissue cultures (test tubes containing minced 10 day chick embryo tissue suspended in 5.0 cc. of Tyrode's solution) with exudate from the sinuses of the 5 birds employed in the infectivity tests resulted in the isolation of one pure strain of the coccobacilliform bodies. This strain was carried through a number of subcultures and compared with a stock culture originally obtained from the type II coryza. The morphology, cultural behavior, and infectivity of the two strains were practically identical.

TABLE I

The Reaction to Type III Exudate after Recovery from Infection with Hemophilus gallinarum

Bird No.	Incubation period	Duration of discharge	Interval to reinfection	Incubation period	Examination of exudate: *H. gallinarum*	Examination of exudate: Coccobacilliform bodies	Examination of exudate: Infectivity
	days	*days*	*days*	*days*			
1	3	13	14	18	–	+	Coryza on 16th day
2	2	6	22	13	–	+	" " 23rd "
3	2	5	23	21	–	+	" " 19th "
4	1	9	20	21	–	+	" " 22nd "
5	1	8	21	28	–	+	" " 20th "

Coccobacilliform bodies were generally demonstrable, thereafter, in birds infected with type III exudate. In a few birds, particularly those with a coryza of long standing, a diagnosis was impossible because of the huge numbers of miscellaneous bacteria. The morphological characteristics of these bodies are so definite that cultivation is not necessary for their identification. The microscopic examination, in addition to demonstrating that coccobacilliform bodies were present in type III exudate, brought out the unexpected finding that they were present as early as the 1st day after injection.

It was now established that exudate from birds infected with the coryza of rapid onset and long duration contained both *H. gallinarum* and the coccobacilliform bodies. It was demonstrated, moreover, that both agents were present throughout the entire course of the

disease. This observation was contrary to the accustomed behavior of the two agents when injected in pure culture. Several years' experience with different strains of the specific bodies had indicated that their development in the nasal passages of the host was slow. They were rarely demonstrable before the 10th day and frequently did not provoke a reaction from the host before the 2nd or 3rd week. In birds infected with type III exudate they were demonstrable as early as the 1st day after injection. Concerning *H. gallinarum* it was known to be recoverable throughout the course of the coryza, but pure cultures were rarely able to maintain their existence in the host for a period longer than 2 weeks and frequently succumbed after a residence of several days. The obvious approach to a solution of this problem was to determine the behavior of an artificial mixture of the two infective agents in the nasal passages of susceptible birds.

The Combined Action of Hemophilus gallinarum and the Coccobacilliform Bodies in Susceptible Fowl

Five successive experiments were carried out on the combined action of the two infective agents. These experiments were unavoidably prolonged, requiring a little over a year for completion. This served, however, to bring them into a rough relationship with the different seasons.

A different culture of *H. gallinarum* was used in each experiment, all of them being isolated from the stock strain of type III coryza. Four cultures of the specific bodies were employed; one isolated from the type III coryza, the others from type II. In preparing suspensions for injection the supernatant was removed from a 24 to 48 hour tissue culture of the coccobacilliform bodies, the sediment finely ground, and suspended in the supernatant. An equal volume of fluid from the base of 24 to 48 hour horse blood agar slants of *H. gallinarum* was added and roughly 0.5 cc. of the mixture injected through the cleft. The infectivity of each component was checked by adding an equal volume of Tyrode's solution and injecting separately. *H. gallinarum* was reisolated at intervals from the birds injected with the mixed suspension and tested. It was often difficult to isolate *H. gallinarum* by plating exudate from birds with a prolonged coryza because of overgrowths by *Bacillus proteus* and other bacteria. If this occurred exudate was injected into a normal bird from which the organism was readily recovered. Films of exudate were also made at intervals and Gram stained for detection of the specific bodies.

All of the 20 birds employed in the five experiments on the combined action of the two infective agents showed a coryza of rapid onset; the incubation periods being 1 day in 11 cases, 2 days in 8, and 3 days in a single instance. The birds were kept under observation for 30 to 80 days and without exception showed a continuous nasal discharge. 3 birds died, after 30, 40, and 70 days. 2 others were so severely affected that they were killed, after 41 and 45 days. In 14 individuals the duration of the coryza was at least 2 months.

TABLE II

Duration of Coryza Produced by Hemophilus gallinarum Alone and in Combination with the Coccobacilliform Bodies

Infective agent	Experiment 1		Experiment 2	
	Bird No.	Duration of coryza	Bird No.	Duration of coryza
		days		*days*
H. gallinarum cultures used in the mixture	1	3	12	5
	2	5	13	7
	3	15	14	4
H. gallinarum and coccobacilliform bodies	4	>45	15	70
	5	>56	16	>70
	6	>56	17	>70
	7	>41	18	>70
			19	>70
H. gallinarum cultures isolated from birds infected with the mixture	8	4	20	5
	9	5	21	4
	10	8	22	5
	11	16	23	10
			24	3

At autopsy all of these birds were emaciated and showed evidence of constitutional disorder. One bird recovered after a nasal discharge which lasted 44 days. With the exception of this case, coccobacilliform bodies were demonstrable at autopsy. *H. gallinarum* was recovered by subinjection from 10 of the birds which showed a nasal discharge for 56 days or longer. The birds which were injected for these isolations also showed coccobacilliform bodies as early as the 1st or 2nd day.

With several exceptions which will be discussed later, the birds injected with *H. gallinarum* alone showed the usual coryza of rapid onset and short duration. In 8 birds the duration was 7 days or less, in 5 it was 10 to 16 days. Most of these birds were killed and autopsied 1 to 2 weeks after recovery. Save for the above noted exceptions the nasal passages and sinuses were normal. Similar findings were obtained with cultures of *H. gallinarum* isolated at intervals from the test birds. The summary of two experiments in Table II affords a comparison of the infectivity of *H. gallinarum* alone and in combination with the coccobacilliform bodies.

Infectivity tests were also made with the several cultures of the coccobacilliform bodies. These tests which were carried out in a separate quarantine unit did not always coincide with the combined experiments in point of time. Some of the birds showed an inapparent coryza which was detected only at autopsy. If a nasal discharge was produced it was always after an incubation period of 2 weeks or longer.

Transmission of the Coccobacilliform Bodies by Indirect Contact

One bird of a group of 4 injected in June, 1937, with a pure culture of *H. gallinarum* continued to show a nasal discharge long after the other 3 had recovered. It was known that *H. gallinarum* occasionally produced a coryza of 3 to 4 weeks' duration, and the case was not regarded as unusual. The bird was reexamined in September, 78 days after injection, and still showed a nasal discharge, though slight and unilateral. On Sept. 13, the discharge having subsided, the bird was killed and autopsied. The nasal passages were normal, but in one sinus there was a large rubbery plug of exudate which showed Gram granules suggestive of the coccobacilliform bodies. A normal bird injected with this material developed a coryza with very distinct specific bodies on the 3rd day. At monthly intervals two additional passages were carried out. All of the birds showed a coryza of at least 8 weeks' duration, the exudate containing both infective agents. Through January, 1938, four additional cases of prolonged coryza were observed in birds originally injected with *H. gallinarum* alone, the specific bodies being again demonstrated in the nasal exudate. Although carriage of the coccobacilliform bodies by indirect contact

had not been observed in the past, it is believed that transfer in this way was responsible for their presence in the birds infected with *H. gallinarum*, development being favored, as it was not in the case of type II coryza, by that organism.

DISCUSSION

The results of the experiment on the injection of type III exudate, following recovery from the coryza produced by *H. gallinarum* in pure culture, are illustrative of a number of such tests made from time to time with different strains. Recovered birds usually showed a temporary resistance to reinfection with cultures of the organism, regardless of the strain. If protection was not afforded, the reinjection of *H. gallinarum* was followed by a coryza of rapid onset and by recovery of the organism from the nasal passages. The injection of exudate was usually followed by a second coryza which in the case of the present strain was characterized by a long incubation period and the absence of *H. gallinarum*. It was impossible to explain this behavior on the basis of a lowered virulence resulting from the artificial cultivation of *H. gallinarum*. The disease produced in the recovered birds closely resembled the type II coryza, and coccobacilliform bodies similar to the etiologic agent of the latter type were ultimately demonstrated in the nasal discharges.

Although the present strain of type II coryza had been maintained by artificial passage for 4 years there was no reason to doubt that both *H. gallinarum* and the coccobacilliform bodies had been present from the beginning. The apparent fact that two infective agents were present in infected birds did not immediately clarify the situation. Both agents were demonstrable throughout the entire course of the coryza, which frequently began on the 2nd day after injection and lasted for at least 2 months. This observation disagreed with the behavior of the two agents in pure culture. The coccobacilliform bodies were rarely demonstrable before the 2nd week following injection and *H. gallinarum* was rarely recoverable after the 2nd week.

Subsequent investigation showed that these two sets of observations were not incompatible. Pure cultures of the infective agents were mixed, and the effect of the mixture compared with that of each component. The pure cultures behaved in the accustomed way;

the mixture resulted in a coryza similar to that produced by type III exudate. The onset was rapid, the course prolonged, and both agents were generally demonstrable throughout its entire course.

The apparent explanation of this situation is that *H. gallinarum* and the coccobacilliform bodies when present together in the nasal passages of the host cooperate in producing an effect that neither is able to accomplish alone. It is clearly a synergistic, or in the older sense (since both agents benefit by the association) a symbiotic reaction. The rapidly multiplying *H. gallinarum* creates a favorable environment for the immediate development of the coccobacilliform bodies. The latter evidently by reason of their tendency to persist in the host prolong the residence of *H. gallinarum*. It is probable that the beneficial influence of *H. gallinarum* is the factor which makes possible the occasional development of the coccobacilliform bodies transmitted by indirect contact, in normal birds the number which gain entrance to the nasal passages being below the threshold for survival.

The cooperative association of *H. gallinarum* and the coccobacilliform bodies adequately accounts for the etiology of the present strain of type III coryza. Schalm and Beach have reported observations which indicate that long continued bird to bird passage of *H. gallinarum*, starting with a pure culture, may prolong its residence in the nasal passages (5). It is possible that a similar adaptation may occasionally occur in nature. Hence, a generalization applicable to this type of coryza is not warranted at present.

SUMMARY

Coccobacilliform bodies were regularly demonstrable, in addition to *H. gallinarum*, in exudate from birds infected with a passage strain of the coryza of rapid onset and long duration (type III). Both agents were present throughout the entire course of the disease.

The characteristics of type III coryza were reproduced by injecting a mixture of the two agents. The behavior of each component was altered by the association, indicative of a synergistic relation.

Evidence that the coccobacilliform bodies might occasionally de-

velop in birds infected with *H. gallinarum* following transfer by indirect contact was also obtained.

The combined action of the two infective agents adequately accounts for the etiology of this particular strain of type III coryza.

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THE AGGLUTINATION OF PLASMODIUM KNOWLESI BY IMMUNE SERUM

By MONROE D. EATON, M.D.

(*From the Laboratories of the International Health Division, The Rockefeller Foundation, New York*)

PLATES 37 AND 38

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The demonstration of passive immunity in experimental monkey malaria by Coggeshall and Kumm (1, 2) indicates that immune substances are present in the sera of animals with chronic malarial infections and of animals that have been hyperimmunized by superinfection. The nature and mode of action of the protective substances is at present unknown. The existence of precipitins and complement-fixing antibodies in human malaria has been reported, but up to the present time the specific sensitization and agglutination of malarial parasites by immune serum has not been demonstrated. This paper will describe and discuss the significance of a specific agglutination of *Plasmodium knowlesi* by immune serum from monkeys. The immune body concerned in this agglutination apparently has a general similarity to antibacterial and other antibodies.

Using antigens prepared from parasitized red blood cells or from malarial spleens, Thomson (3), Kingsbury (4), and others have obtained fixation of complement by sera from cases of benign and malignant tertian malaria. Generally the reactions were rather weak and pseudopositive reactions with syphilitic sera were observed. Precipitin tests for malaria have been described by Pewny (5), Taliaferro, Taliaferro, and Fisher (6), and Row (7). Taliaferro obtained a large number of positive reactions with malarial sera using an antigen prepared from placentas infected with *Plasmodium falciparum*, but this author was unable to repeat the results later with different antigens and sera (8). Of a different nature is the non-specific seroflocculation reaction in malaria described by Henry (9). The character of the antigen (melanin) and of the reactive substances in the malarial sera excludes the possibility that the Henry reaction is a true precipitation of antigen by antibody (10).

In avian malaria the serum of the bird acquires the ability to reduce the electric

charge of both parasitized and unparasitized red cells. Brown (11) has reported that the reduction in the charge on the red cells is related to the degree of resistance to the infection. This implies a non-specific sensitization of all the red cells, but no agglutination was observed. Malamos (12) observed in the blood of a monkey infected with *P. knowlesi* and treated with atebrin an agglomeration of red cells which affected, during the early stages of the disease, only the parasitized cells. Later an agglomeration of all of the red cells occurred and the animal died, despite the fact that the parasite count had been greatly reduced. The phenomenon was observed in only one of 200 animals.

Agglutination of leishmania and trypanosomes by sera from animals or human beings chronically infected with these organisms has been demonstrated repeatedly. In certain instances auto-agglutination of red cells by these sera was also observed. Leishmania and trypanosomes are agglutinated by normal sera in dilutions lower than the dilutions of immune sera required for agglutination. A summary of the work on serological reactions in various parasitic infections will be found in the book by Taliaferro (8).

Materials

Antigen.—The blood of *rhesus* monkeys dying from acute infection with *P. knowlesi* (1) was collected in citrate by bleeding the animals from the heart. In most cases the parasite count of these animals is between 2,000 and 5,000 per 10,000 red cells. Blood with a lower parasite count is generally not suitable for the preparation of antigen because of the difficulty of obtaining a sufficient proportion of mature parasites. The serum is separated from the cells by centrifugation. The blood is resuspended in saline and centrifuged at low speed, or the cells are allowed to settle by gravity in a cylinder. An upper brown layer containing parasitized cells and leucocytes may then be separated from the unparasitized cells which settle more rapidly. With blood which has stood in the ice box for more than a few hours or with old antigens it is advisable to filter the suspension through cotton and use the settling method to remove pieces of clot or clumps of cells.

The cells to be used for antigen are suspended in saline so that an opacity approximately equivalent to that of a 0.5 per cent suspension of red cells is obtained. The resulting suspension should contain 50 per cent or over of cells with mature malarial parasites. It should be free of clumps as determined by macroscopic and microscopic examination, and it should not contain a large excess of white cells. The leucocytes tend to agglutinate spontaneously and this may be confused with agglutination of the parasitized cells. The antigen suspension should be definitely brown. If the color is predominantly red, too many normal cells and immature parasites are present.

Sera.—Animals were bled from the femoral vein and the serum obtained in the usual manner. Except in the experiment with heated and unheated serum, all sera used for agglutination were heated at 56°C. for one-half hour. Sera were collected from twenty normal monkeys, from five monkeys during the acute stages

of the infection, from ten monkeys a few weeks after the acute infection had subsided, from five monkeys with chronic malaria, and from ten superinfected monkeys. The sera from chronically infected and superinfected monkeys were the same as those used in the experiments of Coggeshall and Kumm (2). The animals were superinfected by nine or more injections of 5 cc. each of heavily parasitized blood given at intervals of 7 to 10 days. Sera from five monkeys having chronic infections with *P. inui* of various durations were also tested for agglutination of *P. knowlesi*.

Method of Performing the Agglutination Test

Macroscopic Agglutination.—Diluted or undiluted serum in amounts of 0.2 cc. is pipetted into small tubes 0.8 cm. in diameter and 9.0 cm. long. The suspension of parasitized cells (0.3 cc.) is then mixed with the serum and the tubes allowed to stand at room temperature for 2 hours. Occasional gentle agitation during the first 15 minutes facilitates the agglutination. The agglutinated parasitized cells appear as small granules at the bottom of the tube. In the negative controls the red cells should settle compactly to the bottom of the tube and no granules should be visible. With long standing the granules of agglutinated cells may be obscured by unagglutinated cells which have settled around them. After examination of the bottoms of the tubes for granules the cells are resuspended by gentle shaking and the presence or absence of visible granules or floccules in suspension is noted.

Microscopic Agglutination.—A drop of the mixture of parasitized cells and serum is removed from the tube and examined on a microscope slide under a cover slip with the high power objective. In a positive test clumps of parasitized cells containing pigment granules are visible but there should be no clumping of unparasitized cells. If the reaction is non-specific clumps of both parasitized and unparasitized cells will be seen. Normal serum should give no microscopically visible agglutination.

The hanging drop technique may also be used for performing the microscopic agglutination test. A drop of diluted or undiluted serum is mixed with a drop of the suspension of parasites on a cover slip, then inverted over a hollow ground slide and the preparation sealed with paraffin oil. With strongly positive sera agglutination of the parasitized cells is visible in 15 to 30 minutes.

The macroscopic method just described will detect agglutinins in all but a few of the weakest sera when a good antigen is obtainable. Unless the antigen contains over 50 per cent of mature parasites it may be difficult to see the agglutinated clumps with the naked eye because they are obscured by unparasitized cells which do not agglutinate. With the microscopic technique agglutination may be seen in a suspension containing as little as 5 to 10 per cent of parasitized cells. Non-specific agglutination of red cells and spontaneous clumping of red or white cells can best be distinguished from agglutination of the parasitized cells by examination under the microscope.

Agglutination Titers of the Sera of Immune Monkeys

The sera of twelve monkeys with chronic infections of various durations were tested for agglutination at dilutions up to 1:4,096. Eight of these monkeys had been superinfected repeatedly and their sera, in

TABLE I

Agglutination of Plasmodium knowlesi by Immune Serum from Chronically Infected and Superinfected Monkeys. Titer of Agglutinins

Monkey No.	Infection and duration		Dilution of serum					
			1:4	1:16	1:64	1:256	1:1,024	1:4,096
		mos.						
B2	Chronic,	5	++	++	+	+	±	−
B9	"	2	++	+	+	−		
3-9	"	5	++	+	±	−		
4-0	"	5	+	±	−	−		
5-4	"	2	±	−	−	−		
9	Superinfected,	9	+++	++	++	+	±	−
1-2	"	8	+++	++	+	±	−	−
8	"	11	++	+	+	+	±	−
3	"	9	+++	++	++	+	±	−
4	"	10	++	+	+	±	−	−
7	"	10	++	+	+	−	−	−
2	"	13	++	+	+	−	−	−

Controls: 20 normal sera tested undiluted against the antigen used in these tests gave no agglutination

+++, strong macroscopic agglutination, large clumps of parasitized cells visible in suspension.

++, moderate macroscopic agglutination, clumps definitely visible in suspension.

+, small clumps barely visible in suspension, but definitely visible as granular sediment in the bottom of the tube and under the microscope.

±, agglutination detectable only as slight granular sediment in the bottom of the tube and as small clumps of parasitized cells under the microscope.

−, no agglutination detectable microscopically.

general, possessed stronger protective properties than the sera of the five animals which had not been superinfected (1, 2). The results presented in Table I indicate that the sera of the group of superinfected monkeys agglutinated the parasites at higher dilutions than the sera

of the group of chronic monkeys which had not been superinfected. Four of the sera, three being from superinfected animals, gave definite microscopic agglutination at a dilution of 1:1,024. All the sera from superinfected animals and two of the five sera from chronic infections gave macroscopic agglutination at a dilution of 1:64. In the second column of the table the duration of the infection in months is given. It will be noted that the serum of monkey B2, which had an infection of 5 months' duration without superinfection, gave as high an agglutination titer as the sera of the superinfected animals. Four other monkeys in this group had infections of shorter duration than the superinfected animals and their sera agglutinated at considerably lower titers. Further work with a larger series of monkeys will be necessary before the relationship of superinfections and duration of infection to the strength of agglutination can be definitely determined.

Development of Agglutinins in the Sera of Monkeys during Recovery from Acute Infection

Agglutination of *P. knowlesi* by the sera of normal monkeys taken before infection has not been detected. The sera of five monkeys taken at the height of the acute infection when the parasite count was over 1,000 per 10,000 red cells also gave no agglutination. The blood of these animals had contained demonstrable parasites for 5 to 7 days. After the fall in the parasite count which follows treatment with quinine or immune serum, the sera of most of the monkeys so far tested have shown agglutination.

Table II shows the rise in the titer of agglutinins in the serum of a monkey treated with quinine. At 15 days, after recovery from the acute infection, weak microscopic agglutination was detected. 2 weeks later the agglutination was stronger and during a series of relapses in the next 2 months the titer of agglutinins definitely increased. After 3 months the animal no longer had relapses with high parasite counts and the agglutination titer had reached a relatively high level.

The sera of eight monkeys which had been treated by daily injections of 2 cc. of immune sera for 10 days after the injection of parasites were also tested for agglutination. In this case the presence of passive immunity makes the detection of agglutinins in the early stages of the disease less significant because the agglutination may be due to the

injected immune serum. The results presented in Table III show that five of the eight sera agglutinated the parasites 23 and 33 days after infection. At 44 and 57 days all of the sera gave positive

TABLE II

Development of Agglutinins during Recovery from Acute Infection with Plasmodium knowlesi by a Monkey Treated with Quinine

Time after infection	Dilution of serum				
	Undiluted	1:4	1:16	1:64	1:256
days					
0 (quinine)	−	−	−	−	−
15	±	−	−	−	−
29	+	±	−	−	−
45	++	++	±	−	−
55	++	++	±	−	−
67	++	+	−	−	−
77	++	+	−	−	−
88	++	++	+	±	−
101	++	++	+	±	−

TABLE III

Agglutination of Plasmodium knowlesi by the Sera of Monkeys Recovering from Acute Infection after Treatment with Immune Serum

Monkey No.	Serum	Days after infection			
		23	33	44	57
5-6	Undiluted	−	−	+	
5-8	"	±	±	+	±
5-9	"	+	+	±	+
6-0	"	−	−	±	±
6-1	"	−	−	+	+
6-2	"	+	+	+	+
6-5	"	+	+	±	±
6-6	"	±	+	±	±

agglutination. In addition to these, the sera of two monkeys which recovered from the disease without treatment agglutinated parasites after infections of 18 and 44 days, respectively. These animals repre-

sent an exception to the general rule that untreated infection with *P. knowlesi* is fatal to *rhesus* monkeys.

Tests for Cross-Agglutination of Plasmodium knowlesi by the Sera of Monkeys Chronically Infected with Plasmodium inui

In order to determine whether a chronic infection with another species of malarial parasite causes the production of agglutinins for *P. knowlesi*, sera from monkeys with chronic infections with *P. inui* were tested against the *P. knowlesi* antigen. The duration of infection in the five monkeys tested was 2, 3, 8, 11, and 13 months, respectively. None of the sera gave any agglutination detectable by microscopic examination. Experiments using an antigen prepared from *P. inui* have not been done because the low parasite count in monkeys infected with this species makes the preparation of a satisfactory antigen difficult.

Individual Variations in the Sensitivity of Antigens

Because of the lability of the antigen used in the agglutination tests described in the previous sections of this paper, it has not yet been possible to prepare standardized antigens of uniform sensitivity. Differences in the sensitivity of various preparations of antigen may be attributed to aging, partial clotting of the blood, percentage of parasitized cells, stage of development of the parasites, and possibly individual differences in the cells of different *rhesus* monkeys. With sera which give weak positive reactions these variations may be great enough to determine whether the reaction is positive or negative, but with definitely positive immune sera all the antigens tested have given positive results. In Table IV are presented the results of agglutination of eight separate antigens by ten immune sera. Weak sera, such as Nos. 2, 7, 1-4, and 3-9, give moderate agglutination with all the antigens tested, while relatively stronger sera give corresponding uniformly strong reactions. Antigens D and G may be considered relatively insensitive while antigens C, E, and F are quite sensitive and give strong agglutination with most of the sera. This table also shows that the agglutination of the parasitized cells is apparently independent of the possible existence of blood groups or other individual differences in the blood of monkeys.

Occasional Agglutination of Normal Red Cells by Normal and Immune Sera

During the experiments on the agglutination of the parasitized red cells the positive sera were examined repeatedly for agglutination of normal red cells. This was done both by mixing the sera with the cells of normal monkeys and examining for macroscopic agglutination,

TABLE IV

Agglutination of Separate Preparations of Antigen from Eight Monkeys Dying of Acute Infection with Plasmodium knowlesi

Serum No.	Antigens							
	A	B	C	D	E	F	G	H
Immune sera undiluted								
2	x	x	x	+	++	++	x	x
3	+++	+++	x	++	+++	++	++	++
4	+++	x	+++	++	++	+++	+	x
5	++	++	++++	x	x	x	x	+++
7	+	x	x	x	++	++	x	+
8	++	x	+++	x	++	++	++	x
9	+++	++	x	x	+++	+++	++	++
1-2	++	+++	x	x	++	+++	+	++
1-4	+	x	++	x	x	x	x	x
3-9	x	x	x	+	+	++	x	++
Normal sera undiluted								
N1	—	x	—	x	—	—	—	—
N2	—	—	—	x	x	x	x	x
N3	x	—	x	—	—	x	x	x
N4	x	—	x	x	—	—	x	x

x, not done.

and by examining mixtures of parasitized blood and immune serum under the microscope for agglutination of unparasitized cells. One of the immune sera not included in the preceding tables regularly gave macroscopic agglutination of normal cells from three of four other monkeys. A few other sera, both normal and immune, occasionally gave slight clumping of normal red cells visible under the microscope. This suggests the possible existence of iso-agglutinins

in the blood of *rhesus* monkeys although these have not been found by other investigators (13, 14).

Characteristics of the Material Which Agglutinates

The suspension of parasitized cells used in the agglutination test rapidly deteriorates on standing in the ice box. Spontaneous hemolysis which affects first the parasitized cells occurs after 24 hours. The parasites liberated by hemolysis tend to coalesce into a mass which is not readily dispersed. Attempts to preserve the suspensions in solutions of 25 per cent glycerol, 12 per cent dextrose, or 1 per cent formalin have been unsuccessful. Formalin fixes the parasitized cells but destroys their ability to agglutinate after 24 hours in the ice box. When kept in 25 per cent normal monkey serum the parasitized cells remain agglutinable for as long as 6 days but the sensitivity of the antigen is considerably less than that of a fresh suspension.

The possibility that the agglutination is due to a soluble and readily diffusible antigen on the surface of the red cells was considered. If this were the case, washing should remove the antigen and make the parasitized cells inagglutinable. However, washing the parasitized cells four times with saline slightly diminishes but does not abolish their agglutinating ability.

As may be seen in Figs. 1 and 2, the cells containing the small rings do not agglutinate as readily as the red cells with mature parasites. This indicates that the antigen does not become accessible to the antibody until the parasites have grown to such a size that they fill most of the red cell. In the clumps of agglutinated cells a clear margin around each parasite is generally visible (Fig. 2). This shows that the membrane of the red cell has not been broken. Possibly the growth of the parasite inside the red cell finally damages the cell membrane to such an extent that it becomes permeable to antigen, antibody, or both. The increased fragility of the parasitized red cells, as compared with the unparasitized cells in the same sample of blood, lends support to this view.

Parasites liberated from the red cells by spontaneous hemolysis agglutinate in much the same way that they do when inside of the red cell.

A suspension of "free" parasites was obtained from a sample of heavily parasitized citrated whole blood which had stood overnight in the ice box. Many of the cells containing mature parasites had gone to pieces but there was very little hemolysis of the unparasitized cells. By centrifuging, the free parasites were readily separated in an upper layer, washed once in saline, and resuspended in normal serum. In a smear of this suspension stained by Giemsa it was seen that some of the mature segmenters stained a deep blue, a normal staining reaction of live malarial parasites, but many of the parasites were stained a lighter shade of lavender (Fig. 3). These latter were probably damaged or dead parasites.

The suspension of free parasites was completely agglutinated to medium sized clumps by immune sera but this antigen was not more sensitive than an antigen prepared from unhemolyzed parasitized

TABLE V

Effect of Heat on the Agglutinins in Immune Monkey Serum

Serum No.	Temperature and time of heating	Dilution of serum and agglutination of *P. knowlesi*				
		1:4	1:16	1:64	1:256	1:1,024
B2	Not heated	++	++	+	±	−
"	56°C. for 60 min.	++	+	+	±	−
"	65°C. for 30 min.	+	+	±	−	−
"	75°C. for 30 min.	±	−	−	−	−
3	Not heated	++	++	+	+	±
"	56°C. for 60 min.	++	++	+	+	±
"	65°C. for 30 min.	+	+	±	±	−
"	75°C. for 30 min.	−	−	−	−	−

cells. On staining the agglutinated free parasites it was seen that both the lavender and the blue staining parasites had agglutinated (Fig. 4). This indicates that it is not essential for the parasites to be alive in order to agglutinate. The material which agglutinates apparently has a staining reaction similar to the nuclear material of the parasites.

Effect of Heat on the Agglutinin for Plasmodium knowlesi

The immune body concerned in the agglutination of *P. knowlesi* resembles other antibodies in its behavior to heat. The results presented in Table V show that the agglutinin is not appreciably affected by heating at a temperature of 56°C. for 1 hour, is partly

inactivated at 65° for 30 minutes, and is almost completely inactivated by a temperature of 75° for 30 minutes.

DISCUSSION

The specific agglutination of *Plasmodium knowlesi* by immune serum suggests that a specific sensitization of the parasites may occur *in vivo* and this renders them more susceptible to phagocytosis by macrophages of the spleen and by other pbagocytic cells. The identity of sensitizing and agglutinating antibodies for bacteria has been clearly demonstrated. Since some of the immune sera agglutinate the parasites at dilutions as high as 1:1,000 it is probable that as little as 1 cc. of these sera injected into an infected animal could produce a sensitization of the parasites in the blood stream. The appearance of agglutinins in the sera of monkeys after the recovery from the acute phase of the infection with *Plasmodium knowlesi* also suggests that the relative immunity of these animals is associated with the presence of sensitizing antibodies in the blood stream. The data presented in this paper are not considered to be extensive enough to demonstrate conclusively a relationship between agglutinins and passive and active immunity to malaria but further investigations along these lines will be carried out.

Findlay and Brown (15) have shown that during an attack of avian malaria the degree of non-specific sensitization of parasitized and unparasitized red cells by serum *in vitro* (as measured by the amount of reduction of the electric charge on the red cells) is correlated with the size of the spleen and with the rate of phagocytosis of infected red cells by the macrophages of the spleen. This non-specific sensitization, which is apparently due to an increase in the euglobulin fraction of the serum (11), is not to be confused with the specific sensitization and agglutination observed in monkey malaria. Since agglutination of normal red cells by the serum of one monkey with chronic malaria has been observed, it is possible that non-specific factors do play a part in the agglutination and phagocytosis of parasitized cells, but in monkey malaria these non-specific effects are not as prominent as the specific agglutination.

That specific factors do exist in other forms of malaria, including that of birds, is indicated by the fact that tolerance of one species

of malarial parasite does not confer protection against another species. As Findlay and Brown have pointed out, if the serum plays a part in this specific protective mechanism, the reaction between parasite and serum as a surface phenomenon can probably occur only during the short extracellular stage of the parasite. This is borne out by the observation that the red cells containing the immature ring forms of *P. knowlesi* do not agglutinate. On the other hand, the segmenters which are undoubtedly intracellular do agglutinate despite the apparent intervention between the parasite and the antibody of the membrane and cytoplasm of the red cell. Increased permeability of the membrane has been offered as a possible explanation for this. In view of this observation it seems likely that the parasites may be susceptible to the immune effects of the serum over a longer period of their life cycle than was assumed by Findlay and Brown.

At the present stage of the work the value of the agglutination test as a diagnostic measure in malaria is diminished by the difficulties of preparing a suitable antigen. The sera of a number of human cases of paresis which had been treated by infection with monkey malaria were tested against an antigen prepared from the cells of an infected monkey. In no case was it possible to differentiate between non-specific agglutination of all the red cells, due to hetero-agglutinins in the human sera, and specific agglutination of the parasitized cells. Suitable material for the preparation of antigen from human blood infected with tertian, sub-tertian, or quartan malaria has not been available.

SUMMARY

A specific agglutination of *Plasmodium knowlesi* detectable both by macroscopic and by microscopic methods is described.

Agglutinins for *Plasmodium knowlesi* appear in the sera of monkeys between 15 and 45 days after the onset of the infection and become progressively stronger as the malarial infection gradually subsides.

Agglutinins persist in the sera of chronically infected animals for a year or longer. The sera of animals which have been repeatedly superinfected agglutinate parasites at dilutions as high as 1:1,000.

Sera from normal monkeys, from monkeys acutely ill with malaria, and from monkeys chronically infected with a different species of

malarial parasite (*Plasmodium inui*) do not agglutinate *Plasmodium knowlesi.*

Immune serum agglutinates mature intracellular or extracellular parasites but does not agglutinate unparasitized cells or cells containing immature parasites.

The relation of these observations to the mechanism of active and passive immunity in monkey malaria is discussed.

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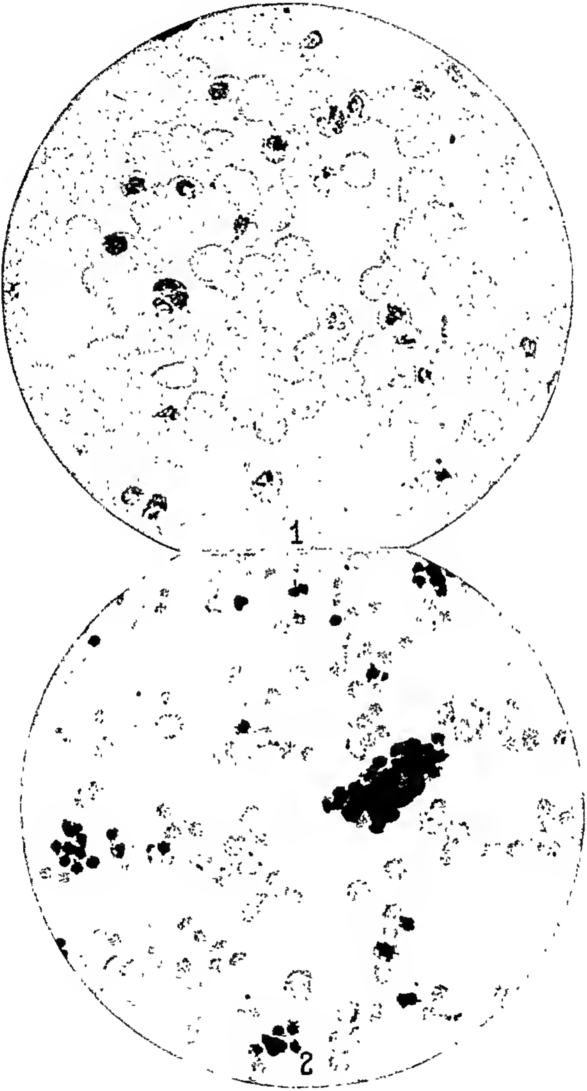
EXPLANATION OF PLATES

Plate 37

Fig. 1. Red cells containing *P. knowlesi* at various stages of development, showing absence of agglutination in normal monkey serum. × 820.

Fig. 2. Agglutination by immune serum of red cells containing mature forms of *P. knowlesi*. In the clumps of parasitized cells a lightly stained zone around each parasite represents the remaining cytoplasm and membrane of the red cell. Cells containing immature parasites, visible in the photograph as small dots, are not agglutinated. The clumps of parasitized cells include no unparasitized red cells. × 820.



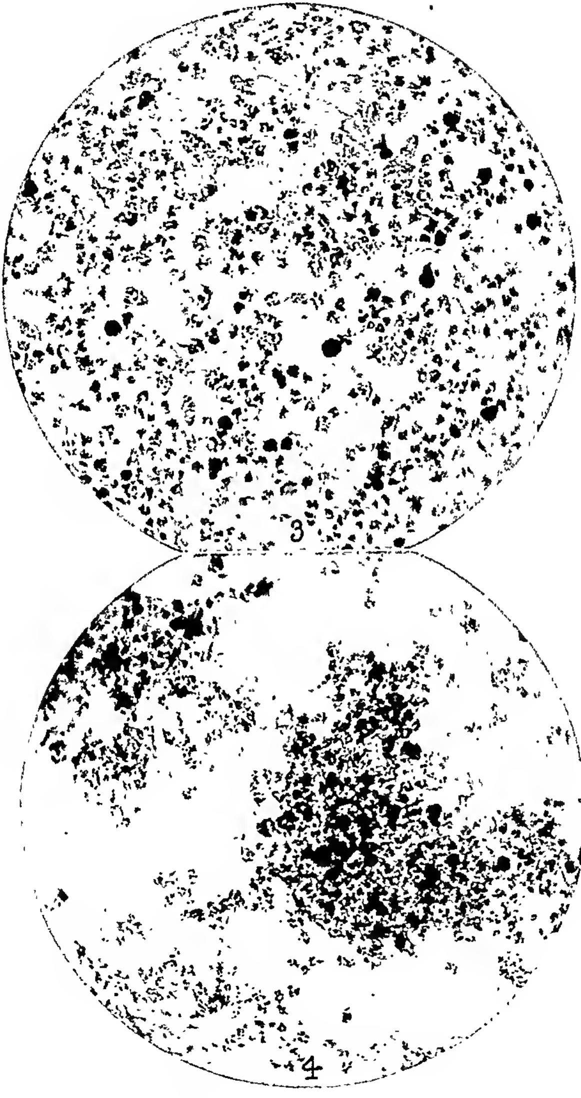
Photographed by Joseph B. Haulenbeek

(Eaton: Agglutination of *Plasmodium knowlesi*)

PLATE 38

FIG. 3. Free parasites resulting from spontaneous hemolysis of parasitized cells 24 hours after drawing the blood. Parasites suspended in normal serum. The large, round, darkly stained (blue) forms are mature segmenters. Lighter and irregularly stained (lavender) forms are probably damaged or dead parasites containing pigment granules. The suspension contains very few intact red cells. $\times$ 820.

FIG. 4. Agglutination of free parasites by immune serum. The clumps contain both light and dark staining forms. Practically all of the material has agglutinated. $\times$ 820.



Photographed by Joseph B Haulenbeek

(Eaton: Agglutination of *Plasmodium knowlesi*)

THE COMPLEMENT FIXATION REACTION IN MONKEY MALARIA

By LOWELL T. COGGESHALL, M.D., and MONROE D. EATON, M.D.

(*From the Laboratories of the International Health Division, The Rockefeller Foundation, New York*)

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A specific complement fixation reaction for malarial infections has given negative results in the hands of several workers (1–3), while with others (4–8) positive findings have been obtained. In some instances failure to obtain specific reactions seems to have been due largely to faulty technique. At the present time there is no accepted complement fixation test which is adaptable for routine use in the investigation of malarial infections. The chief difficulty encountered has been the lack of any means whereby it would be possible to obtain a standardized specific antigen. Thomson (5), in an attempt to overcome this difficulty, used a modification of Bass and Johns' (9) method of cultivation in order to obtain an antigen with a larger proportion of parasitic material. He obtained rather weak complement fixation with this material. Kingsbury (8) utilized different internal organs and heart's blood of human beings with heavy *Plasmodium falciparum* infections in an attempt to obtain an antigen of low anticomplementary power but with high specificity and sensitivity. Saline emulsions of heavily parasitized heart's blood proved to be most effective, yet the sera from 25 known cases of *falciparum* malaria fixed complement in only 48 per cent of the instances. Manson-Bahr (10) felt that a very specific antigen could possibly be obtained from the oocysts on the stomachs of infected mosquitoes. He was unable to demonstrate any complement-fixing activity with the alcoholic extract of emulsified stomachs, but the material he had to work with was limited.

No noteworthy studies dealing with the complement fixation in malaria have appeared following the above reported contributions. However, in view of the promising results obtained by some of the workers, in spite of the lack of satisfactory source of antigenic material, it seemed likely that the intense infections of *Plasmodium knowlesi* in *rhesus* monkeys would provide an abundant source of parasites for the preparation of a suitable antigen. The present study deals with the procedures employed in the preparation of various antigens and the method of performing the test. The results with sera of monkeys

tested during the acute and chronic stages of *P. knowlesi* infections indicate that specific antibodies appear in the early stages of the disease and persist during the course of the subsequent chronic infection. The variations in titer may be correlated with the presence or absence of circulating parasites.

Materials and Methods

Antigen.—The material used for antigen in the complement fixation test was prepared from the spleen or the blood of monkeys dying of infection with *P. knowlesi*. Three types of antigen have been used.

1. The infected spleens were chopped up, frozen, and dried in the frozen state in a vacuum desiccator. The dried material was then ground in a ball mill at −70°C. according to the technique described by Mudd *et al.* (11). The ground material was extracted overnight in the refrigerator with 10 cc. of saline for each gram, and the insoluble residue centrifuged down. The supernatant was used for antigen.

2. Blood containing 20 to 50 per cent of parasitized red cells was collected in 2 per cent sodium citrate solution, centrifuged to separate the serum, and washed twice with saline. The packed red cells and parasites suspended in an equal volume of saline were then frozen, dried, and ground by the same procedure as that described for the spleens. The antigen was prepared by extraction with saline.

3. The parasitized blood, washed as described above, was mixed with three volumes of distilled water, toluene was added for a preservative, and the material was allowed to autolyze in the incubator for 48 hours. The insoluble residue was centrifuged down and the supernatant used for antigen after adding enough salt solution to bring it to isotonicity. Antigen was also prepared by adding three volumes of distilled water to parasitized blood which had undergone autolysis in the refrigerator for several weeks, and then centrifuging.

The antigens prepared from blood or spleens by the methods just described do not greatly differ in the complement fixation reaction with immune sera. The antigens prepared from blood are slightly more sensitive than those prepared from spleen and have been used in all but two or three of the series of tests described in this paper. Several other methods for preparing antigen have been tried. The material extracted from infected spleen by acetone, alcohol, or ether does not give a specific fixation of complement with immune serum. The material extracted from spleens or blood with acid or alkaline buffers is not a better antigen for complement fixation than that extracted by saline or distilled water. Attempts to separate the antigen from inactive protein by tryptic digestion were unsuccessful because the antigen was rapidly destroyed.

The antigens were titrated for anticomplementary and hemolytic properties. In the test a dilution of antigen was used which was at least 4 times the dilution showing slight anticomplementary effects, and 8 times the dilution showing

marked anticomplementary action. In most cases this was a dilution of the original antigen solution of 1:10 to 1:16. The antigens did not have any marked hemolytic activity.

Sera.—Monkeys were bled at regular intervals of 7 to 10 days, and the serum was inactivated and stored in the ice box. At intervals of 6 to 8 weeks complement fixation tests were done on the series of specimens collected at different dates from each animal. Successive samples of serum were collected from seven monkeys beginning shortly after the recovery from the acute phase of the malarial infection, from three monkeys during the chronic infection with frequent relapses, and from ten superinfected monkeys (12) with long standing infections and infrequent relapses.

Method of Performing the Complement Fixation Test.—The hemolytic system consisted of 5 per cent sheep cells and anti-sheep rabbit serum. The unit of amboceptor was taken as the smallest amount which produced complete hemolysis in the presence of an excess of complement (fresh or frozen and dried guinea pig serum). The complement was titrated on each day before the tests were set up, using 2 units of amboceptor. Complement having a unit greater than 0.15 cc. of a dilution of 1:10 was discarded. The complement fixation test was set up as follows:

0.2 cc. serum undiluted, 1:2, 1:4, 1:8, etc.

2½ units of complement (0.35 cc. or less of 1:10 dilution).

0.25 cc. antigen diluted as described.

Controls:

1. Normal serum with antigen and complement.
2. Immune serum undiluted, 1:2, 1:4, 1:8, etc., with complement and 0.25 cc. saline in place of antigen (serum control).
3. Antigen at dilution used in test with complement and 0.2 cc. saline in place of serum (antigen control).

Incubate for 1 hour at 37°C. in the water bath and add 0.5 cc. of a mixture of equal volumes of 5 per cent sheep red cells and amboceptor diluted so that 0.25 cc. contains 2 units.

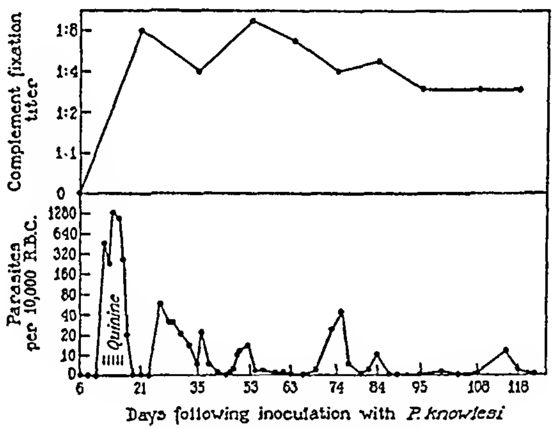
After adding the hemolytic system the tests are read as soon as the controls have cleared, usually between 15 and 30 minutes. Since monkey sera, undiluted or diluted 1:2, are frequently anticomplementary, the serum controls may not clear completely. In this case the test is read after 30 minutes if the controls with antigen and normal serum show complete hemolysis.

Because of the anticomplementary properties of monkey serum, it is necessary to sacrifice some sensitivity in the test by using 2½ units of complement instead of 2 or 1½ units. The excess of complement may cause weak reactions to fade quickly so that it is necessary to follow the progress of hemolysis rather closely. On the other hand, reactions with strongly positive sera do not change appreciably after long standing.

The titers of the sera in the tests are recorded as the highest dilution of serum which gave a fixation of complement demonstrable by the presence of unhemolyzed

red cells. In the graphic representation of the titers used in this paper, the location of the points on the graph was sometimes determined by interpolation. For example, if a serum gave a +++ reaction at 1:8 and a ± at 1:16, the point was placed midway between 1:8 and 1:16 on the graph. If the reaction was + at 1:8, the point was placed exactly at 1:8, but if the reaction was ++ at 1:8 and − at 1:16, the point was placed slightly above 1:8. When the sera were definitely anticomplementary, it was also necessary to make allowance for this. Thus when a serum gave a reaction equivalent to ++ or greater at 1:2 in the serum control without antigen, the titer was considered to be half of the figure indicated by the test with antigen. When the sera were anticomplementary at 1:4, the test was considered unsatisfactory and the results have not been used.

Parasite Counts.—The intensity of the infection and the occurrence of relapses were determined by making parasite counts in blood smears from the infected



TEXT-FIG. 1. Monkey B9.

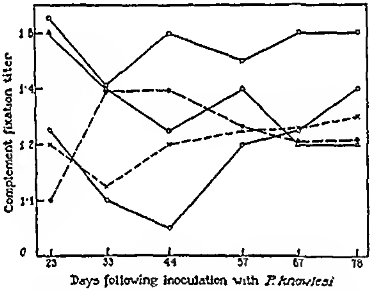
monkeys at intervals of 1 to 3 days. These are recorded as the number of parasitized cells per 10,000 red blood corpuscles.

Appearance of Complement-Fixing Antibodies in the Sera of Monkeys after Recovery from Acute Infection

The changes in parasite count and the complement fixation titer of the serum of monkey B9 treated with quinine are shown in Text-fig. 1. At 21 days, when the animal had recovered from the acute infection, the complement fixation titer was relatively high. Between 21 and 35 days the titer fell despite the constant presence of parasites in the blood. At 53 days the titer had risen again after a period of low grade infection followed by a relapse. In the next 2 months the

titer gradually fell despite the occurrence of three definite relapses with intervening low grade infection.

The titers of the sera of seven monkeys which had been treated by daily injections of 2 cc. of immune serum for 10 days after the injection of parasites were also followed in a similar manner. The results for five of the monkeys are shown in Text-fig. 2. Two monkeys in this group had traces of complement-fixing antibodies between the 23rd and 78th days after infection, but since their sera were quite anticomplementary, the results are not included in the graph. Four of the curves are similar in form to that shown in Text-fig. 1. At 23 days the titer was high, and this was followed by a decline to the 33rd day.



TEXT-FIG. 2. ●, monkey 5-8; ○, monkey 5-9; ×, monkey 6-0; □, monkey 6-4; △, monkey 6-5.

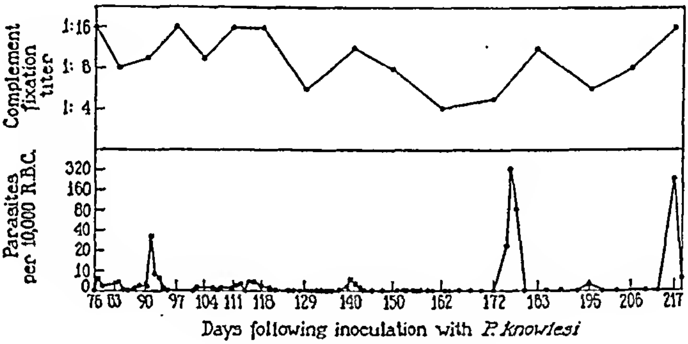
It is possible that part of the complement-fixing antibodies found in these sera on the 23rd day came from the injected immune serum, the last dose of which had been given 13 days previously. One of the sera (No. 5-8 from a monkey which also received immune serum) showed a low titer on the 23rd day, followed by a rise.

The secondary rise in titer and the subsequent gradual leveling off seen in the curve for the serum of the monkey treated with quinine may also be seen in four of the curves in Text-fig. 2 in the corresponding stages of the infection. The somewhat different response in monkey 5-8 may have been due to the fact that parasites did not appear in the blood of this animal until the 13th day after infection,

while the others became positive for parasites between the 4th day and the 8th day. The two monkeys which developed only traces of complement-fixing antibodies had infections which were as heavy and prolonged as those of the other animals.

Changes in the Complement Fixation Titer during the Chronic Relapsing Phase of Plasmodium knowlesi Infection

As indicated in the preceding section, the complement fixation titer of the sera of monkeys infected with *P. knowlesi* reaches a relatively constant level 2 to 3 months after the animals have recovered from the acute infection. This level is apparently maintained by the occurrence of repeated relapses with the appearance of a considerable



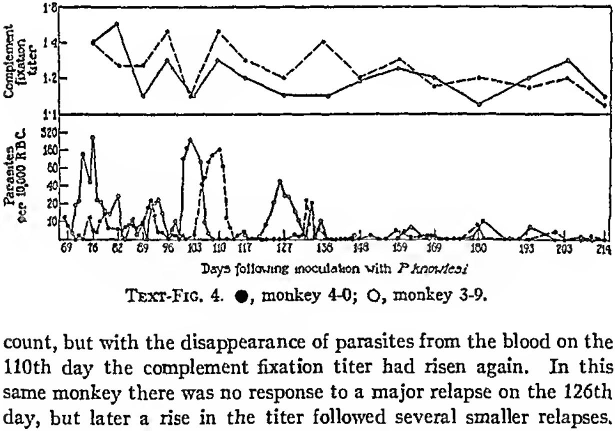
TEXT-FIG. 3. Monkey B2.

number of parasites in the blood. Different monkeys show wide individual variations in the level at which the complement-fixing antibodies are maintained.

During the chronic stage of the infection the rise and fall of the complement fixation titer of the serum appears to be related to the occurrence of relapses. This is illustrated in Text-figs. 3 and 4. Monkey B2 (Text-fig. 3) during the period of observation had three major relapses, on the 91st, 177th, and 217th days, respectively, and three minor relapses, on the 104th to 118th days, the 140th, and the 196th days, respectively. A fall in the curve for complement fixation titer preceded each of the six relapses, and a rise in the curve accompanied or closely followed each relapse. Since the points for the complement fixation titer are placed on the graph at intervals of 7

to 12 days, while the points for the parasite count are placed at intervals of 1 to 3 days, an exact conformity of all parts of the curves cannot be expected. For this same reason it is possible that some of the higher peaks on the complement fixation curve may have been missed.

Similar results are shown in Text-fig. 4, but here certain exceptions may be noted. Between the 76th and 89th days the sera of the two monkeys showed a fall in complement fixation titer which was accompanied by the almost constant presence of parasites in the blood. Between the 89th and 96th days peaks in the parasite count were followed by peaks in the curves for complement fixation. In monkey 3-9 on the 103rd day a fall in the curve followed a rise in the parasite



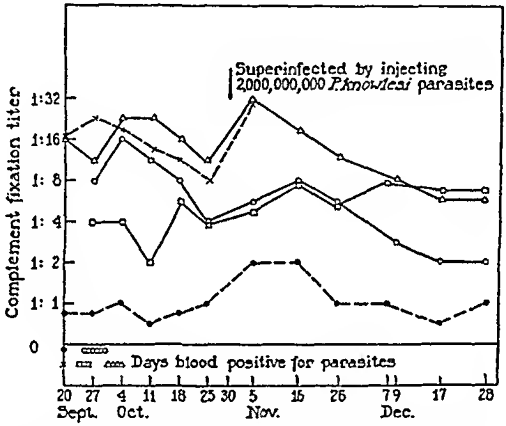
TEXT-FIG. 4. ●, monkey 4-0; O, monkey 3-9.

count, but with the disappearance of parasites from the blood on the 110th day the complement fixation titer had risen again. In this same monkey there was no response to a major relapse on the 126th day, but later a rise in the titer followed several smaller relapses. The curves for monkey 4-0 show a relation between parasite count and rise and fall of complement fixation titer similar to that for monkey B2 (Text-fig. 3).

Effect of Superinfection on the Titer of Complement-Fixing Antibodies

A group of ten monkeys with chronic malarial infections ranging in duration from 8 to 13 months were tested at regular intervals over a period of 2 months for complement-fixing antibodies. Each of the

group of monkeys in Text-fig. 5 had a relapse some time during the period of 2 weeks from September 20 to October 4, and in each case the relapse was followed by a rise in the complement fixation titer, except in monkey 4 in which the rise was preceded by a slight fall in titer. In these animals the relapses are seldom severe. Frequently only one to five parasites per 10,000 red corpuscles are found over a period of a few days. This seems to be sufficient to cause as great a rise in the complement fixation titer as a more severe relapse in a monkey having chronic malaria of shorter duration (compare Text-figs. 4 and 5).

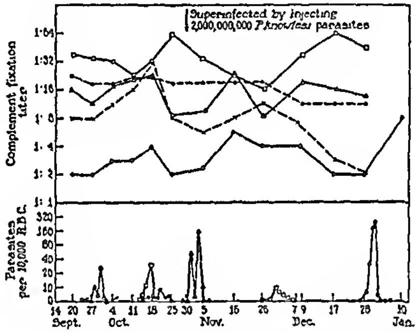


TEXT-FIG. 5. ●, monkey 2; ○, monkey 3; ×, monkey 1-4; □, monkey 4; △, monkey 5.

On October 30 each of the monkeys in the group was superinfected by the injection of blood containing approximately 2 billion parasites into the peritoneal cavity. All five animals had responded by November 5 with an increase in complement fixation titer. This rise in titer persisted in monkeys 2, 3, and 4 for 2 weeks and in No. 5 for only 1 week. Monkey 1-4 died as a result of bleeding. Following the rise after the superinfection, the curves tend to fall to constant levels.

The results with the other five monkeys in this group are shown in Text-fig. 6. Three of these monkeys each had one or more relapses of moderate severity. Monkey 7 had relapses on the 18th of October and 30th of November, each of which was followed by a sustained rise in the complement fixation titer, but this monkey failed to respond

with increased titer to the superinfection which was given shortly after a relapse. Monkey 1-2 showed no rise in titer as a result of a relapse on October 23 and superinfection a week later. In this monkey the level of complement-fixing antibodies remained relatively constant over the period of 2 months. Monkey 6 had a relapse on October 1 and another on the 1st to 5th of November immediately after superinfection. Each of these relapses was followed by a rise in the complement fixation titer. This monkey also showed a secondary rise on December 7 which was not accompanied by demonstrable parasites in the blood. Monkeys 8 and 9 showed rises in comple-



TEXT-FIG. 6. ●, monkey 1-2; ○, monkey 8; × monkey 9; □, monkey 7; △, monkey 6.

ment fixation titer following mild relapses and after superinfection, thus resembling in behavior the five monkeys in Text-fig. 5. Monkey 8 also had a severe relapse on December 30 which was followed by a sharp rise in the curve. In all of these curves there is a definite indication that the complement fixation titer tends to fall off gradually when the blood remains free of parasites for a long period of time.

DISCUSSION

In general, the best antigens were obtained from blood containing a high proportion of mature parasites, or from spleens taken from

monkeys with intense infections. In order to extract the antigen in a soluble form, it was apparently necessary to break down the parasites as completely as possible. It was found that freezing, drying, and grinding the parasitic material or permitting it to autolyze was sufficient to liberate a soluble antigen extractable with normal saline solution. Since the antigen was not extractable by lipoid solvents and was destroyed by tryptic digestion, the active principle behaves as a protein.

Monkeys treated in the acute stage of the infection with quinine or immune serum showed a similar response in the production of complement-fixing antibodies. The titer rises promptly after an acute attack and then proceeds to drop. There is a secondary rise which tends to level off, and subsequently the titer, whether high or low, seems to remain at a rather uniform level for any particular animal. During the chronic stage of the infection when major and minor parasitic relapses appear at irregular intervals, the titer is low immediately preceding a relapse and is elevated after the relapse has terminated. The severity of the relapse appeared to have no correlation with the complement-fixing titer. The continued presence of large numbers of parasites in the blood as the result of repeated relapses apparently may produce an effect on the titer opposite from that observed when relapses appear at longer intervals of time. The fall in complement fixation titer observed in those monkeys with frequent relapses is probably due to the removal of circulating antibody or to the exhaustion of the mechanism responsible for antibody production.

There is a wide variation among different monkeys in the degree of production of complement-fixing antibodies. Some animals maintain the titer at relatively high levels; others produce practically no antibodies detectable by complement fixation. Similarly, some animals show a prompt and extensive rise after a relapse or superinfection while others show a delayed response or none at all. The sera used in this study were taken from monkeys which had previously served as the source of serum in the demonstration of protective antibodies (13). It was noted in some animals that the complement fixation titer was low and the titer of the protective antibodies was high; also the sera of others having high complement fixation titers showed

little protective effects. However, in the individual animals any factor which influences the level of protective antibodies may also influence the corresponding level of the complement-fixing antibodies. This assumption has been experimentally demonstrated in monkeys which have been tested for complement-fixing and protective antibodies immediately before and after relapses and will be described in more detail in a later report. By analogy there is also an apparent relationship between these two antibodies, as a relapse is followed by an increase in the titer of complement-fixing antibodies; and as the animal is able to overcome the relapse spontaneously, there must be an increase in the concentration of protective antibodies. There is no evidence to show that complement-fixing antibodies and protective antibodies are identical.

SUMMARY

1. A specific complement fixation reaction test for *Plasmodium knowlesi* malaria in *rhesus* monkeys is reported with details involved in the preparation of the antigen and procedures employed in setting up the test.

2. It was found that specific complement-fixing antibodies appeared early in the course of the experimental disease and persisted during the course of the chronic infection.

3. The first appearance of complement-fixing antibodies was generally followed by a temporary fall in titer. During the first 2 months of infection there was no apparent relationship between the number of circulating parasites and the changes in complement fixation titer.

4. During the stage of chronic infection there was a fall in the titer of complement-fixing antibodies preceding each parasitic relapse, and after the relapse had terminated, there was an elevation of the complement-fixing titer.

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THE FATE OF VACCINIA VIRUS ON CULTIVATION IN VITRO WITH KUPFFER CELLS (RETICULO-ENDOTHELIAL CELLS)

By JOSEPH W. BEARD, M.D., and PEYTON ROUS, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

PLATE 39

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Many facts indicate that the cells constituting the "reticulo-endothelial system" play a rôle in combatting the invasion of the mammalian organism by bacteria and viruses. Much there is also to suggest that such cells are the source of some antibodies at least. The work done on these problems has been inconclusive, however, because it has been impossible to exclude the participation of other elements of the animal body in the responses obtained. The development of techniques whereby the reticulo-endothelial cells lining the liver sinusoids (Kupffer cells) can be isolated in quantity (1) and cultured at will *in vitro* (2) has seemed to provide an opportunity for decisive tests of their abilities. We have attempted to learn whether they have any effect upon vaccinia virus.

General Methods

To obtain the Kupffer cells advantage was taken of their activity in the phagocytosis of foreign particles circulating in the blood stream. A suspension of very finely divided, strongly magnetic iron oxide was injected into rabbits intravenously, and some days later Kupffer cells were dislodged from the liver by perfusion with Tyrode, combined with massage, and they were sorted out of the washings by means of a powerful electro-magnet. For *in vitro* cultivation the cells were transferred to dishes containing lens paper immersed in rabbit serum. They distributed themselves along the fibres of the paper and were kept in good condition by changing the serum often.

The necessary cells and serum were procured from large brown-gray (agouti) rabbits, and animals of this breed were utilized for the ultimate tests of the effects of the cultivated cells upon the virus.

Homologous serum is the medium in which the Kupffer cells fare best. That procured from presumably normal, "agouti" rabbits which have been kept for some

time under the conditions prevailing in the animal house of The Rockefeller Institute not infrequently exerts some neutralizing effect on vaccinia virus exposed to it *in vitro*. For this reason animals freshly procured from breeders were used for serum purposes in the cultivation experiments. By preliminary neutralization tests with serum specimens and vaccinia in high dilution, individuals were selected which yielded serum devoid of neutralizing effect on the virus; and they were bled to death into chilled tubes containing just enough sterile heparin solution to delay clotting until after the cells had been brought down with the centrifuge and the plasma taken off. A bit of sterile, voluntary muscle was now introduced into the latter to precipitate clotting, and by twisting the clot with the aid of a pipette serum free from hemoglobin, or but faintly tinged with it, was obtained. The yield from several animals was pooled, distributed in tubes, and kept in the refrigerator. Each of the experiments was carried out with a single batch of pooled serum. This was employed within a few days, that is to say while alexin was still present. The strain of vaccinia used was that of the New York Board of Health, in the form of glycerolated vaccine lymph.[1] Rabbits were inoculated with it intratesticularly, and after 3 to 4 days the infected tissue was excised, hashed, a portion used for titration tests, and the remainder tubed in 1.0 cc. quantities and frozen for storage. When virus was needed for an experiment a tube was thawed at room temperature, and the tissue was ground with sand, diluted with Tyrode and "decelled," or in other words freed of particulate matter by a method already described (3). The material titrated high, yielding characteristic lesions on intradermal inoculation of 0.2 cc. at dilutions of 1:1,000,000 or more in Tyrode. Throughout the titrations in the experiments calibrated record tuberculin syringes were employed, and 0.2 cc. was injected as the standard amount. Only this quantity was taken up in the syringe for each inoculation, and these were made into the shaved sides of 2 to 4 rabbits, with variation in the arrangement of the several inocula to cancel out the influence of local differences in their position. The lesions they induced were outlined each day with a wax pencil on a superimposed sheet of transparent celluloid and then directly traced on the record cards. Only the findings of the early days are given in the charts, before necrosis had complicated the picture.

Tyrode solution was employed for washing and dilution, and specially calibrated micropipettes for the handling of minute quantities of material.

Immediate Tests with Kupffer Cells

In some initial experiments washed Kupffer cells, freshly obtained by means of the magnet, were mixed with a suspension of virus and injected into the skin of rabbits.

Experiment 1.—A normal rabbit weighing about 2,300 gm. was given intravenously 20 cc. of iron oxide-acacia suspension daily in two injections of 10 cc. on

[1] Kindly provided by Dr. Rivers.

each of 3 successive days, and 72 hours later the cells were flushed from the liver with about 300 cc. of Tyrode, collected on a collodion surface with the magnet, and washed by running about 100 cc. of Tyrode slowly past the brown pellicle of cells while this was still held fast. The magnetization was then discontinued and the material shaken off into 0.9 cc. of Tyrode. Of the resulting turbid, ruddy brown suspension, 0.35 cc. was put into each of two tubes, and one was heated in a water bath at 53°C. for 15 minutes to kill the cells. When it had been cooled to room temperature, 0.01 cc. of 1 per cent virus was added to it, as also to the tube containing the living cells, and to a control with 0.35 cc. of Tyrode, and after 5 minutes of gentle agitation, 0.01 cc. more of virus was introduced into them all. The usual difficulty was experienced in suspending the living Kupffer cells, which are extraordinarily sticky, soon forming clumps that cannot be broken up. In heated suspensions this does not happen. All of the tubes were agitated for 10

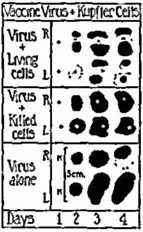

CHART 1. In this and the succeeding charts the lesions indicated by dotted lines were pink swellings slightly raised above the skin surface; those represented in black were elevated, purplish, indurated plateaus or mounds. Cross-hatching signifies necrosis.

minutes more at room temperature; then 0.6 cc. of Tyrode was added to all; and intradermal inoculations were made forthwith. 2 test rabbits were employed, with intradermal injections of all three materials on each of their sides.

One of the animals proved extremely susceptible to the action of the virus, enormous, confluent lesions resulting from the inocula. Chart 1 shows the results obtained in the other animal. The control mixture of Tyrode and virus gave rise to large areas of swelling, acute inflammation, and eventual necrosis such as vaccinia ordinarily causes, and so too did the material containing killed cells. Where the inoculum of living cells had been put the situation of the injected cells could be readily discerned by reason of the iron in them, which produced a brown spot several millimeters across. For some distance around this, the skin remained normal (Chart 1), but further away lesions developed like those caused by the other inocula but much smaller, and with less tendency to necrose.

In this experiment the virus injected together with the living Kupffer cells gave rise to relatively small lesions, and while the difference in the results with the inocula seemed significant the possibility that serum antibodies had been carried over into the mixture with the cells was not wholly excluded by the washing to which they were subjected. The following experiment was carried out in the same way except for the introduction of fresh normal serum into the tubes prior to the addition of virus. The activity of Kupffer cells for bacterial phagocytosis in normal animals is known to depend largely upon the presence in the blood of a thermolabile principle, or principles, an opsonin, so called. A very little fresh serum enables them to take up bacteria perfused through the liver (4).

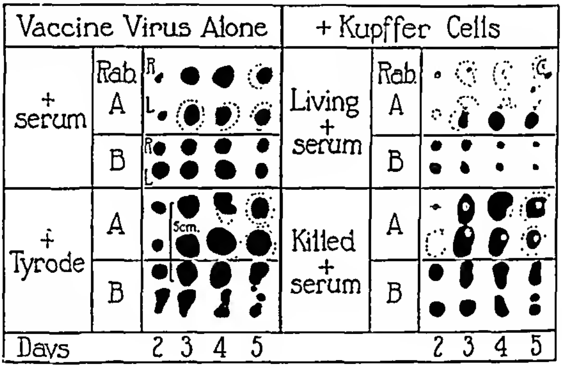

CHART 2

Experiment 2.—An especially large yield of Kupffer cells was procured from a normal rabbit, and they were thoroughly washed with more than 100 cc. Tyrode while held with the magnet, and distributed in portions of 0.35 cc. to two tubes, one of which was heated as in Experiment 1. To each tube there was then added by means of a micropipette 0.04 cc. of fresh normal rabbit serum, previously cleared of any cells with the centrifuge, and after the mixtures had been shaken briefly 0.02 cc. of 0.4 per cent virus was added, after which the tubes were gently shaken at intervals during 30 minutes at room temperature. Two control tubes had been set up containing 0.35 cc. Tyrode plus 0.04 cc. serum and Tyrode respectively, and 0.02 cc. of virus. After all had stood for 30 minutes at room temperature intradermal injections were made into each side of 2 test rabbits.

The results of this test are depicted in Chart 2. It will be seen that the serum added to the mixtures had some neutralizing influence on the virus, the lesions produced by the control material containing it being smaller than those from the

control with Tyrode only. The heated Kupffer cells had no effect, whereas the presence of living ones, together with serum, resulted in an almost complete suppression of the virus activity in one rabbit (B), which developed mere dubious, transitory thickenings of the skin, while in the other rabbit lesions appeared only at a distance from the brown spot where the Kupffer cells lay, just as happened in Experiment 1.

In several further experiments of the sort, similar results were obtained. For one of them Kupffer cells were employed from an animal recently recovered from vaccinia. They had been wasbed as usual and they neutralized the virus no more effectually than did the cells from normal rabbits.

Tests with the Cells of Peritoneal Exudates

The experiments were now extended to determine the effect of other cells of the reticulo-endothelial system. The chief representatives of this system, outside of the liver, spleen, lymph nodes, and bone marrow, are the clasmatocytes. It is common knowledge that these cells largely replace the polymorphonuclear leucocytes which at first assemble after the injection of a sterile irritant into the peritoneal cavity. This happens, as we have found, when the injected material is an iron-acacia suspension of the sort used to procure Kupffer cells from the liver. After 72 hours clasmatocytes largely predominate in the exudate. They can be obtained from the exudate in considerable quantity and they proved much more hardy than Kupffer cells, living many hours in Tyrode solution, as evidenced by the trypan blue test of viability (5). For the experiments which follow, 72 hour and 24 hour exudates were utilized, the one clasmatocytic, that is to say consisting mostly of large mononuclear cells, with clasmatocytes predominating, the other polymorphonuclear, consisting almost entirely of such elements. Differential counts were done as routine. The clasmatocytes had taken up much more iron.

Experiment 3.—Rabbit A, which had recovered from an experimental intradermal infection with vaccinia 21 days previously, was injected intraperitoneally with 10 cc. of iron-acacia suspension on 2 successive days. Another recovered animal, B, of the same lot, received in a single injection 20 cc. of the suspension. On the morning of the experiment, that is to say 72 and 24 hours, respectively, after the last injection of the two animals, they were bled to death from the heart into containers in which 2 cc. of heparin (1:1000 in Tyrode) was already present. From rabbit A 60 cc. of blood was obtained, from B, 45 cc., and after centrifugation of it the leucocytic pellicles were taken off practically intact, washed 3 times in

19 cc. of Tyrode, and finally suspended in 0.8 cc. Meanwhile the abdomens of the animals had been opened aseptically and the peritoneal exudates washed out with 50 cc. of Tyrode in each case, containing 2 cc. of 1 per cent heparin. Examination with neutral red of the material thus obtained from rabbit A, which had been injected 72 hours before, revealed great numbers of clasmatocytes and a few monocytes, most of them containing iron particles in greater or less quantity. Not many polymorphonuclear cells were present, but some of these held one or several fine iron particles. To reduce the number of polymorphonuclear cells still

TABLE I

	Material		Serum	Virus	Iron	Heated iron	Tyrode
		cc.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
Rabbit A	Living blood leukocytes	0.35	0.04	0.02			0.7
	Heated " "	"	"	"			"
	Living " "	"	"	"	0.33		0.38
	Heated " "	"	"	"		0.33	"
	Living exudate cells (mostly clasmatocytes)	"	"	"			0.7
	Heated " "	"	"	"			"
Rabbit B	Living blood leukocytes	"	"	"			"
	Heated " "	"	"	"			"
	Living " "	"	"	"	"		0.38
	Heated " "	"	"	"		"	"
	Living exudate cells (mostly polymorphonuclears)	"	"	"			0.7
	Heated " "	"	"	"			"
	Iron suspension	"	"	"			"
	Heated iron suspension	"	"	"			"
	Tyrode solution	"	"	"			"
	" "	0.39	—	"			"

further the suspension was passed by the magnet, and the iron-containing elements thus sorted out were washed *in situ* with more than 100 cc. of Tyrode.

The exudate from rabbit B, recently injected, consisted predominantly of polymorphonuclear cells carrying little or no iron. It was centrifuged and the deposit was washed by centrifugation in 3 changes of 40 cc. each Tyrode. While this was being done, the particles from 2 cc. of the standard iron-acacia suspension were washed 3 times in Tyrode, and a 0.4 per cent suspension of vaccinia virus was prepared. On the preceding day serum from a normal rabbit had been procured, and some of it heated at 53° for 10 minutes. Mixtures were now made as listed in Table I. The cell materials were first distributed to the tubes in the order

given, then serum was added, with agitation, and then virus, the whole taking about 10 minutes, after which the tubes were gently agitated by hand for 20 minutes at room temperature. Iron suspension, heated or unheated, plus Tyrode, or Tyrode alone, was then introduced as indicated, and the injection was begun at once of 0.2 cc. portions of the various mixtures into 4 rabbits. All the inoculations with each mixture were carried out at one time, in the order of their prepara-

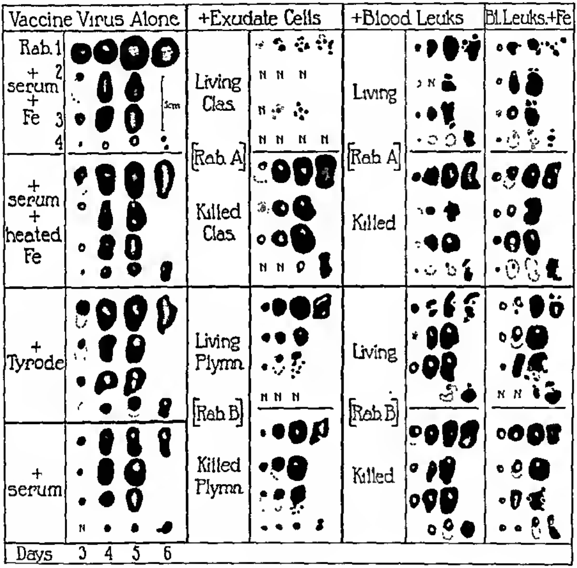

CHART 3

tion, and the intervals were such that the mixtures had been in existence for approximately the same period prior to their injection. In 2 of the animals some of the lesions coalesced after the 5th day. Consequently these are not charted thereafter.

It will be seen from Chart 3 that virus plus Tyrode caused pronounced lesions in all of the animals. When normal serum was also present somewhat smaller lesions resulted. Virus plus normal serum

plus iron caused lesions larger, if anything, than those due to virus alone, and the same held true when the iron had been heated. Virus plus serum plus blood leukocytes, heated or unheated, with or without iron (some of which was promptly phagocyted by the unheated cells), gave rise in general to lesions nearly as large as those produced by the control of virus plus serum. Evidently the blood leukocytes, though derived from immune animals, had but a slight effect on vaccinia under the circumstances of the experiment. This was true as well of the cells of the more recent of the two exudates, that in which polymorphonuclear elements predominated. The heated cell material of the older, clasmatocytic exudate seemed actually to favor the virus as compared with the other materials. This was not true of the living, unheated clasmatocytic material, however. It acted to neutralize the virus almost or quite completely in contrast to all the other inocula. Furthermore, in those instances in which lesions did arise they were situated at some distance from the brown spots marking the location of the cells, just as had happened in Experiments 1 and 2.

In the next experiment, similar exudate materials were utilized, from rabbits which had not been subjected to vaccinial infection, and the conditions were varied in other ways. Aleuronat instead of iron acacia was used to induce exudate formation, inactivated as well as fresh serum was employed, and no iron was added to any of the mixtures. The blood leukocytes were not tested.

Experiment 4.—2 normal rabbits, A and B, were injected intraperitoneally with 5 cc. each of a sterile suspension of aleuronat in starch solution, 24 and 72 hours respectively before the test was to be carried out. The exudates were washed from the peritoneal cavity in the same way as in Experiment 3 and the cells were thrown down with the centrifuge, washed twice with Tyrode, and made up to 1.6 cc. Counts showed them to be mainly polymorphonuclear leukocytes in one instance and clasmatocytes in the other. 0.5 cc. of the original aleuronat material was washed twice in the same way.

The 2 rabbits providing the exudates had been bled 5 cc. each on the previous day. The sera thus procured were united in equal amount, after they had been twice centrifuged to exclude cells, and a portion was heated at 56°C. for 30 minutes. Mixtures were then made with a 0.2 per cent suspension of virus, as shown in Table II. All of the mixtures were gently agitated at intervals by hand for 30 minutes at room temperature, prior to injection of 0.2 cc. portions into 4 rabbits. The order of preparation and injection was as given in the table.

It will be seen from Chart 4 and Figs. 1 and 2 that the control material of virus plus Tyrode gave rise to large lesions in all the animals. The control with heated serum caused equally large ones, but there was a slight reduction in their size when unheated serum was used instead. The cells of the exudate that consisted mostly of polymorphonuclears had some adverse effect upon the virus, and this was more considerable when the cells had not been killed by heat; but whether the serum was fresh or heated made no difference in the result.

TABLE II

	Material		Serum	Heated serum	Virus	Tyrode
		cc.	*cc.*	*cc*	*cc.*	*cc.*
	Aleuronat suspension	0.35		0.04	0.02	0.7
	Heated aleuronat suspension	"			"	"
	Aleuronat suspension	"			"	"
Rabbit A	Living exudate cells (mostly clasmatocytes)	"		"	"	"
	Heated " "	"		"	"	"
	Living " "	"	0.04		"	"
	Heated " "	"	"		"	"
Rabbit B	Living exudate cells (mostly polymorphonuclears)	"		"	"	"
	Heated " "	"		"	"	"
	Living " "	"	"		"	"
	Heated " "	"	"		"	"
	Tyrode solution	"	"		"	"
	" "	"		"	"	"
	" "		—	—	"	"

As in Experiment 3, large lesions resulted from the mixtures containing the killed material of the clasmatocytic exudate; and again it seemed to be immaterial whether the serum was heated or fresh. The living clasmatocytes, however, had a pronounced effect to suppress the action of the virus, and in the presence of unheated serum this was nearly or quite complete. The results with the aleuronat mixtures are not charted since the size of the lesions showed the substance to be wholly devoid of effect on the virus.

In another experiment (Chart 5), carried out in a precisely similar way but with aleuronat exudates from animals that had recovered from experimental vaccinial infection 20 days previously, the living material that was predominantly polymorphonuclear in content suppressed the virus to the same extent as did that which was mostly clasmatocytic, and this whether the normal serum added was fresh or had been heated. Control mixtures of virus with the last wash

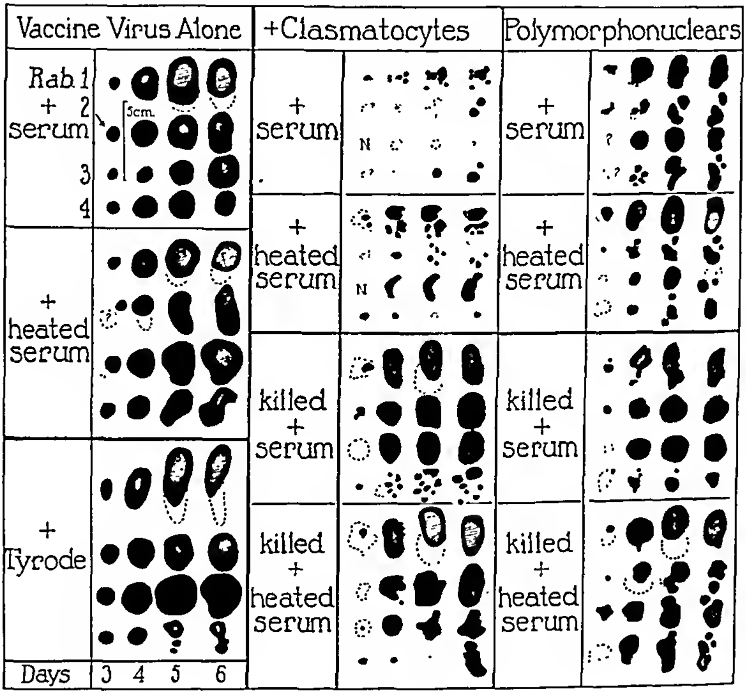

CHART 4

fluid from the cells proved the latter to be devoid of effect. The routine examination of the polymorphonuclear exudate with neutral red had shown its cells to be, as usual, nearly all of this type; and the reason for its effectiveness,—which constituted a difference from the previous findings,—is not clear. The total bulk of polymorphonuclears in the mixtures with virus was somewhat larger than in the case of the clasmatocytes, but this had been true in the other experiments also.

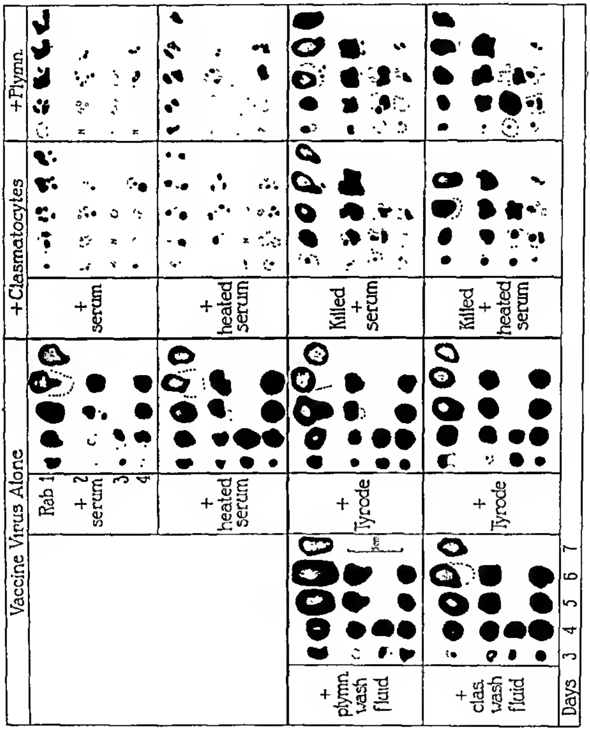
Vaccine Virus Alone
+Clasmatocytes
+Plymn.
Rab 1
+ 2
serum
3
4
+ heated serum
+ Tyrode
+ serum
Killed + serum
Killed + heated serum
+ plymn wash fluid
+ clas. wash fluid
Days 3 4 5 6 7

CHART 5

The Fate of Vaccine Virus in Cultures of Kupffer Cells

In the work just described the conditions had been simplifie removal of the reticulo-endothelial cells from the body for the pu of *in vitro* mixture with the virus and serum; yet while the tests still in progress fresh complications were brought into play by introduction of the materials into the skin of new hosts. To a this difficulty resort was now had to determinations of the fat vaccine virus in cultures of Kupffer cells *in vitro*. Two experim of the sort will be reported which are representative of the res obtained in several others of less comprehensive character.

Following methods already described (1, 2), the Kupffer cells were sorted with the magnet and washed with Tyrode, suspended in homologous serum, transferred to flat dishes containing two layers of lens paper. They clambered on the fibres of this paper, distributed themselves along its fibres, and were read maintained in immense number by replacing the serum every 24 to 48 hours. has been found that under such circumstances the cells do not become fatty show other evident degeneration during 10 days at least, though no significa increase takes place in their number. When the serum is diluted with Tyrode t cells fare less well.

In the cultures employed for the present experiments Kupffer cells were s abundant that the layers of paper on which they were maintained appeared rust brown, owing to the iron particles contained within them. A few monocytes an polymorphonuclear leukocytes that had taken up such particles, and hence bee attracted by the magnet, were always present at first in the cultures, but within 2 or 3 days the polymorphonuclears died. The fate of the monocytes is uncertain, since cultivated Kupffer cells come to approximate them in morphology after a week or more has elapsed. Many of the cells contained very little iron, and the excellent condition of those holding more was shown by the alacrity with which they took up and segregated neutral red when exposed to it in slide and cover slip preparations after a week of cultivation or longer. The phagocyted iron particles were not visibly broken down or dissolved during the period of cultivation. Indeed they have been found in their first abundance, and with the ferromagnetic character retained, in the Kupffer cells of animals killed many months after their injection into the blood stream.

Experiment 5.—The iron suspension employed to procure the Kupffer cells for this test, and for Experiment 6, consisted of 4 gm. of gamma ferric oxide and 14 gm. of gum acacia, twice the ordinary amounts, in 1,000 cc. of distilled water. It was found to be of considerable advantage to keep the suspension in the ice box, autoclaving the necessary quantity just prior to use, since autoclaving reduces the capacity of the gum to hold up finely divided material for long periods of time.

The Kupffer cells from a normal rabbit injected intravenously with the suspension in the usual way were collected, washed with Tyrode while still held by the magnet, and suspended in 2 cc. of a freshly prepared mixture of 4.8 cc. pooled normal serum with 1.2 cc. of 1 per cent virus. Carrel dishes 3 cm. in diameter were employed for the cultures, each dish containing two discs of lens paper that covered its bottom. Since some iron particles always escaped from the cells during the manipulations, control cultures were set up containing the same or a slightly greater amount of such particles from the original suspension, washed free from acacia. Six dishes were prepared as follows, all with lens paper, and incubated at 37°C.

Dish A. 0.6 cc. cell suspension in serum-virus mixture + 1.4 cc. pooled normal serum.

B. 0.6 cc. cell suspension in serum-virus mixture + 1.4 cc. Tyrode.

C. Like B.

D. 0.6 cc. serum-virus mixture as such + 1.4 cc. serum + trace washed iron.

E. 0.6 cc. serum-virus mixture as such + 1.4 cc. Tyrode + trace washed iron.

F. Like E.

The following mixtures, standards for eventual titration of the virus in the cultures, were preserved in sealed pyrex tubes at −5° to −8°C.:—

G. 0.6 cc. serum-virus mixture as such + 1.4 cc. serum.

H. 0.6 cc. serum-virus mixture as such + 1.4 cc. Tyrode.

I. Several cc. of the 1 per cent virus suspension.

Daily 1.7 cc. of fluid, practically all that could be procured, was withdrawn with a pipette from each of the cultures. It came away water clear but was centrifuged to remove the few cells present, transferred to a tube, and stored in the freezing chest with the virus standards already there. To replace it a like amount of the original batch of pooled normal serum was added to cultures A and D, and serum plus Tyrode in the proportion of 6:14 to cultures B, C, E, and F. Each daily change involved dilution of the culture virus with new material in the ratio of 3:17.

The cells as seen under the microscope in the warm box appeared very active. Soon after the plating into whole serum they distributed themselves upon the fibre of the lens paper in the customary way (2). In the serum-Tyrode, on the other hand, most of them remained grouped, and soon many had rounded up and some appeared dead. None of the fluids was changed on the 5th day. On the 6th the cells cultured in serum appeared as numerous as before and showed no fat globules. Many in the Tyrode culture, B, had dropped from the fibres, obviously dead, and this culture was discarded; but those in C were in better state though with some fatty granulation. On the 6th day the culture fluids were pipetted off as usual; the lens papers were withdrawn from A and C; the cells were shaken free from the fibres into Tyrode, so far as this was possible, and washed twice, each time with 45 cc. of it, and made up to 0.75 cc. for injection. Many could not be dislodged from the lens fibres and hence were lost.

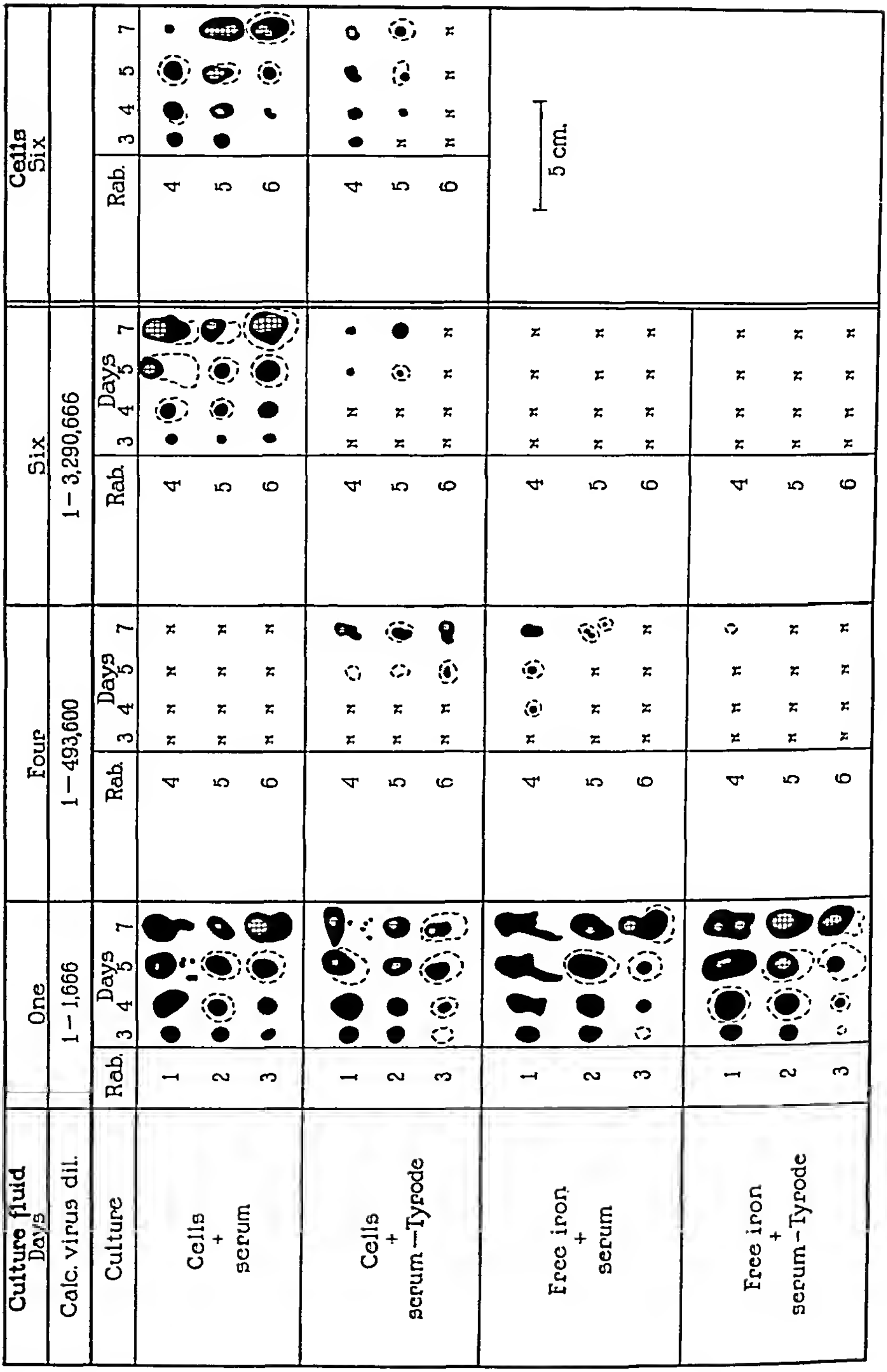
Culture fluid
Days
Calc. virus dil.
Culture
One
Four
Six
Cells
Six
1 – 1,666
1 – 493,600
1 – 3,290,666
Rab.
Days
3 4 5 7
Cells
+
serum
Cells
+
serum – Tyrode
Free iron
+
serum
Free iron
+
serum – Tyrode
5 cm.

CHART 6

Approximately 0.3 cc. of culture fluid had been present with the cells when they were shaken into the Tyrode. The theoretical dilution of virus free in the culture fluid and carried with it through the progressive replacements to the final cell inoculum was of the order of 10^{-12}.

The fluids from A, C, D, and F, that had been stored after 1 and 4 days incubation were now thawed and injected intradermally in 0.2 cc. quantities into 3 rabbits, and so too were the final, or 6 day, fluids and the controls G and H. Material I, diluted repeatedly just as the virus had been in the cultures, but with Tyrode, was also injected. Similar inoculations were made into 3 other rabbits after dilution of the materials to 1:10 with Tyrode.

Vaccine virus of the strain employed retains its activity unimpaired for many weeks when in the frozen state. Just prior to inoculation the frozen control specimen of virus (I) was thawed, and diluted repeatedly in accordance with the successive culture dilutions. In our experience no virus-serum mixture has ever given larger lesions on immediate injection than has a similar mixture with Tyrode alone. In the present instance Tyrode had to be employed as the diluting medium instead of the stock serum, since the latter had been used up. The dilution sufficed to attenuate the virus so greatly that it caused no lesions, a finding which is not charted.

In this experiment (Chart 6) a considerable quantity of virus was added to the fluids in which the cells were maintained during the first 24 hours, and thereafter most of these fluids were almost entirely replaced on several occasions. Appropriate control preparations were made to determine the fate of the virus when no cells were present. The reason the incubation was kept up for 6 days and the fluid replacements repeated, was to get rid by dilution or inactivation of the virus originally free in the fluid, and to afford time for the cells to elaborate an antiviral principle in case this was one of their functions. The fluid introduced into the cultures after 4 days was left on them for 48 hours with a view to the accumulation in it of antiviral principles.

The cells nourished by whole serum flourished, showing no signs of the degeneration to be noted in connective tissue cultures into which vaccinia has been introduced (3). Only the usual few cells came away at the pipetting off of each day, and when the test was terminated the generality were in excellent state, free from fat, segregating neutral red promptly, and clinging so fast to the lens paper that many could not be dislodged by vigorous shaking in Tyrode. The cells in the dish receiving serum diluted with Tyrode, on the other hand, did as badly as usual in such a medium, but no worse than when no

virus was present. The successive replacements of the latter had been sufficient on calculation to have rendered it ineffective by dilution, and whether for this reason or because of inactivation during incubation the fluid removed from the control cultures on the 6th day failed to cause lesions.

All of the fluids removed from the cultures after one day of incubation gave rise to pronounced and characteristic lesions, those from the cultures containing the cells being slightly smaller than from those that had none, as might have been expected had some of the virus been taken out of the fluid by fixation on the cells. That the serum, as such, had no neutralizing effect upon the virus of significance in the present relation was indicated by the fact that the culture with whole serum yielded as large lesions as the one in which it had been diluted with Tyrode in the proportion of 14 to 6.

After 4 days of incubation, with daily replacement of the culture fluid to so great an extent that the initial virus quantity underwent a calculated dilution to about 1 in 500,000, two of the fluids failed to give rise to lesions, and two others, one from a culture with cells, yielded very small and late ones. These results can be explained on the assumption that the virus had been diluted or incubated into inactivity: there is no need to invoke any participation of the cells. After 6 days both of the cultures devoid of cells failed to yield virus, whereas it was present in the fluid from those containing them, notably in the culture with whole serum which had failed to yield it on the 4th day. This contained many Kupffer cells in excellent condition, and the fluid from it now gave rise to large necrotizing lesions, whereas that from the culture in serum-Tyrode, in which the cells had undergone fatty change, was nearly devoid of activity. Some influence of these degenerating cells to maintain the virus was manifest nevertheless, both the culture fluid and the washed cells themselves causing small, late lesions, whereas the cell-free controls yielded none. The healthy-looking Kupffer cells from the culture in whole serum containing virus gave rise to large, necrotizing lesions on injection after repeated washings. It should be remarked that iron-containing Kupffer cells, as such, living or killed with heat, give rise to no lesions on intradermal injection.

It was plain from the results as a whole that the Kupffer cells, instead of neutralizing vaccinia, had served to maintain it under conditions which led to its disappearance when they were absent. In the

culture in which the cells had done well the virus had actually increased. This cannot have been merely because they provided conditions favorable to such virus as was free in the medium and out of their reach, so to speak. For active virus existed in association with the cells themselves, as proven by the necrotizing lesions that resulted from injection of them after thorough washing with Tyrode.

A more comprehensive experiment was next undertaken in which the Kupffer cells were cultivated for 7 days, with replacement of most of the fluid each day, and with immediate titration of the specimens removed at this time, and comparison with some of the initial serum-virus mixture that had been kept in the frozen state.

Experiment 6.—A very abundant yield of rabbit Kupffer cells, procured as usual, was suspended in 1.5 cc. of a mixture of 3 cc. of 1 per cent virus in Tyrode and 12 cc. of pooled, normal serum; and the following cultures were made:

Dish A. 0.6 cc. of cell suspension + 1.4 cc. of serum-virus mixture.
B. The same.
C. 2 cc. of the serum-virus mixture alone.
D. The same.

The remainder of the serum-virus mixture, distributed in small tubes in 0.5 cc. portions was preserved frozen, and so too was some of the 1 per cent virus.

As in the previous experiment 1.7 cc. of water clear fluid was drawn off from each of the cultures daily, but this time in every instance it was replaced with undiluted normal serum of the original batch. The specimens from the duplicate cell cultures were pooled, centrifuged to remove any cells, and the supernatant fluids taken off; and the controls of serum incubated with lens paper were similarly treated. While this was being done a small tube of the original serum-virus mixture was allowed to thaw at room temperature, and now it and the culture fluids were serially diluted with Tyrode; and when the dilution had on calculation reached 1 in 100,000, 1 in 1,000,000 and 1 in 10,000,000 in terms of the original virus, inoculations were made of 0.2 cc. quantities into 4 normal rabbits, a new group of animals being used each day.

It has just been stated that at each successive replacement of the culture medium the residual 0.3 cc. was diluted by the addition of 1.7 cc. of the original batch of serum. Similar successive dilutions, but with Tyrode, were carried out with the specimens of thawed virus-serum mixture when these were utilized for the control inoculations. In doing this no allowance was made for the Tyrode accompanying the cells when they were first introduced into the cultures as 0.6 cc. of suspension,—whence it follows that the original serum-virus mixture, kept frozen and now utilized for the titrations, underwent less dilution than did the portions of it that had been introduced into the cultures.

After 4 days of cultivation, when the virus in the culture fluid would have had

a titre of 1 in 148,000 if it had persisted throughout the successive dilutions, some of this fluid was injected as such intradermally, for comparison with control material diluted in the same way; and the rest was made to 1 in 1,000,000 and compared with control fluid similarly diluted. For the purpose of the titrations made after 5 days and 6 days, the control material was diluted to 1 in 989,000 and 1 in 6,000,000 respectively and compared with the culture fluids as such; but after 7 days the control was diluted only to 1 in 6,600,000, through inadvertence, as compared with the 1 in 44,000,000 obtaining in the culture fluids.

The results of the comparison at 1 in 100,000 of the culture fluids of the early days are given in Chart 7, while those at 1 in 1,000,000 will be found in Chart 8. Because of the successive replacements of the culture medium, a comparison at the lower dilution could be carried out with the material of only the first 3 culture days. The results of the single set of comparative titrations, made later with the materials of the 5th, 6th, and 7th days, have been inserted into both charts. They are based on the calculated dilutions attained at these times by the virus in the culture, as has been stated in the preceding paragraph.

Some slight differences were encountered in the individual susceptibilities of the 4 normal rabbits injected intradermally each day for the purposes of the titration; but since every animal received all of the materials of the culture day for which it was employed, these did not seriously complicate the findings. The latter are consistent. They show a progressive diminution in the amount of active virus in the fluid procured from the control cultures, which after 6 and 7 days gave rise only to negligible lesions. The fluids that were procured from the cultures with Kupffer cells after 24 and 48 hours exhibited a diminished pathogenicity, as compared with those from the control cultures, as would have happened if some virus had been removed by fixation on the cells; but thereafter the specimens from the cell cultures steadily became more pathogenic, and those procured after 6 and 7 days gave rise to very large, necrotizing, vaccinial lesions. There can be no doubt that the virus had not merely persisted in active form in the cultures with the Kupffer cells, but had undergone so great an increase as to have much more than made up for successive dilutions by replacement that sufficed to render the incubated cell-free control practically innocuous. Unfortunately the frozen portion of the original cell-serum mixture that was utilized for comparison with the material procured from the cultures after 7 days was diluted only to 1 in 6,600,000, not to 1 in 44,000,000 as the latter had been through successive replacements. But though about seven times the stronger,

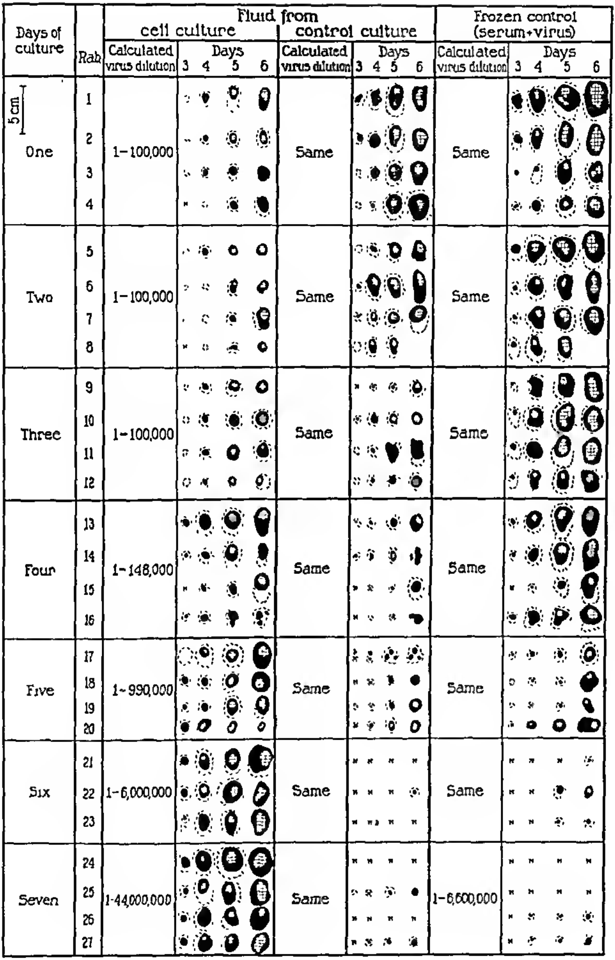
Days of culture
Rab
Fluid from
cell culture
control culture
Frozen control (serum+virus)
Calculated virus dilution
Days
3 4 5 6
5 cm
One
1 2 3 4
1-100,000
Same
Same
Two
5 6 7 8
1-100,000
Same
Same
Three
9 10 11 12
1-100,000
Same
Same
Four
13 14 15 16
1-148,000
Same
Same
Five
17 18 19 20
1-990,000
Same
Same
Six
21 22 23
1-6,000,000
Same
Same
Seven
24 25 26 27
1-44,000,000
Same
1-6,600,000

Chart 7

Days of culture	Rab.	Fluid from cell culture: Calculated virus dilution	Fluid from control culture: Calculated virus dilution	Frozen control (serum + virus): Calculated virus dilution
5 cm. One	1	1-1,000,000	Same	Same
	2			
	3			
	4			
Two	5	1-1,000,000	Same	Same
	6			
	7			
	8			
Three	9	1-1,000,000	Same	Same
	10			
	11			
	12			
Four	13	1-1,000,000	Same	Same
	14			
	15			
	16			
Five	17	1-990,000	Same	Same
	18			
	19			
	20			
Six	21	1-6,000,000	Same	Same
	22			
	23			
Seven	24	1-44,000,000	Same	1-6,600,000
	25			
	26			
	27			

Days of culture	Rab.	Cells A (Days 3 4 5 6)	Cells B (Days 3 4 5 6)
Seven	24		
	25		
	26		
	27		

relatively speaking, it gave rise to negligible changes in 2 animals and to none in 2 others, whereas the culture fluid caused extensive and characteristic lesions in all of them.

During the 7 days of cultivation the cells appeared to be in excellent condition, though at its end a few fatty granules were present in some of them. Now as many as possible were shaken off the lens paper and inoculated intradermally. Inspection of a slide preparation with neutral red showed the generality to be alive, and they segregated the stain actively. Those from each culture were washed twice with about 15 cc. of Tyrode and suspended in 1 cc., with the injection of 0.2 cc. into each of the test rabbits of the final day. Large necrotizing lesions resulted, characteristically those of vaccinia (Chart 8). As a control to the possible presence and influence of minute fragments of lens paper carried along with the cells, the discs of this paper from the cell-free cultures were treated like those from which the cells had been dislodged. Tyrode was forcibly pipetted upon them and, although nothing was seen to come away, the hypothetical residues were centrifuged, made up with Tyrode, and injected like the cells. No lesions resulted.

In a preliminary experiment which calls for only brief mention, the conditions were varied by exposing freshly obtained Kupffer cells to the virus, and then washing and culturing them in serum. On the basis of previous findings which have demonstrated a fixation of vaccinia on connective tissue cells (3), it was assumed that some of it could be fixed upon Kupffer cells, which, after washing, would have only this amount to cope with. The serum employed as culture medium had been inactivated by heat, a procedure known to have no evident adverse effect upon the maintenance of Kupffer cells *in vitro*.

Experiment 7.—30.0 cc. of 1 per cent vaccine virus was mixed with 260 cc. of freshly obtained liver perfusate from an animal previously injected with iron acacia. The perfusate contained many sorts of cells in Tyrode. Selection of the Kupffer cells with the magnet was at once begun. It required 3 hours, and the large yield thus got was washed by slowly flowing 100 cc. of Tyrode past the brown cell layer. The magnetization was then stopped, and the cells were shaken into 3.3 cc. of normal rabbit serum,—which had been inactivated 2 days previously by heating at 56°C. for 30 minutes. Duplicate cultures were prepared, each containing 0.5 cc. cell suspension + 1.5 cc. of inactivated normal rabbit serum.

Each day 1.7 cc. from each culture was replaced with the same amount of inactivated serum. The fluid removed was stored frozen after centrifugation. That taken off after 4 days,—when the cultivation was discontinued,—was pooled, centrifuged, and injected as such into 2 rabbits and also after dilution to 1 in 10 and

1 in 100. At this time the specimens procured after the first 24 hours were thawed and similarly treated. The cells were dislodged from the lens paper by forcible pipetting with a little Tyrode, and those from the two cultures were united, made to a 1 cc. suspension without washing, and injected as such and in the further dilutions just mentioned.

The outcome of this test was essentially the same as in Experiments 5 and 6. The fluid removed from the cell cultures after the first 24 hours incubation, even when undiluted yielded only very small nodular lesions, whereas the specimens obtained on the 4th day gave rise to large and characteristic ones. The biggest lesions caused by any of the materials tested at the end of the cultivation were produced by the cell inocula.

In this experiment the amount of free virus introduced into the cultures must have been very slight; and the considerable increase that took place between the first and 4th days in the quantity present in the fluid media can scarcely be attributed to the release of virus originally fixed upon the Kupffer cells, since the fluid of the first 24 hours incubation yielded small indication of any such release, while thereafter the culture fluids had been six-sevenths replaced on two occasions. The inference seems warranted that events took the same course as in the two experiments already detailed, the virus increasing in association with the cells.

DISCUSSION

The findings leave no doubt that the activity of vaccinia virus is lessened or suppressed when it is mixed in the test tube with living reticulo-endothelial cells (Kupffer cells or clasmatocytes) and injected intradermally after some minutes at room temperature. The effect of these elements is far greater than that of polymorphonuclear cells. In those instances in which some suppression of the virus was observed, in mixtures with the cells of exudates in which polymorphonuclears predominated, clasmatocytes were also present, and they may have been a responsible factor.

Ledingham found that when vaccinia was inoculated into skin that had been injected with India ink, either no lesions developed or small ones situated beyond the edge of the inky patch (6). He concluded that the reticulo-endothelial cells, marshalled or multiplying in the cutaneous tissue as a result of the presence of the ink, were the cause of the virus suppression. Our finding that vaccinial lesions develop only at a considerable distance from reticulo-endothelial cells when

mixed with such cells and injected intradermally adds support to this view.

There are several ways in which the association of the virus with reticulo-endothelial cells might conceivably result in its suppression. The first possibility requiring consideration is that these cells might have failed to survive transfer, and the virus have been destroyed incidentally to their autolysis. As already mentioned, vaccinia becomes fixed rapidly and firmly upon connective tissue cells exposed to it *in vitro;* and Experiment 8 of the present work shows that this happens with living Kupffer cells also, as was to have been expected from their enormous, sticky, surface membranes (2) and activity in phagocytosis.

To learn whether rabbit Kupffer cells will survive transfer to the cutaneous tissue of another individual, as was done in the tests, a suspension of them obtained with the magnet in the usual way was injected intradermally into a new host in the 0.2 cc. amount employed in the experiments, and 18 and 48 hours later the brown spots showing where they lay were excised together with the surrounding skin, fixed in Zenker's fluid and sectioned in series. Under the microscope the precise point of introduction of the material could be readily discerned, for some debris lay there, including a little free iron. The Kupffer cells appeared in excellent condition after 18 hours though still mostly rounded, as when procured with the magnet, and they were at or near the immediate injection site; but after the lapse of 48 hours many had become stellate or lay spread out against connective tissue fibrils, and some had migrated to a considerable distance (Figs. 3 and 4). The course of events was, in other words, remarkably like that when Kupffer cells are introduced into cultures containing lens paper, and they ruled out the possibility that these elements had died *en masse* shortly after introduction into the skin. de Haan and Hoekstra (7) have found that clasmatocytes marked by a content of trypan blue will survive transfer to other hosts.

The Kupffer cells of this experiment had not been exposed to vaccine virus; but Experiments 5, 6, and 7 have sufficiently demonstrated that they flourish in association with it on cultivation *in vitro,* even when the test is so arranged as to insure much preliminary fixation of the virus upon them (Experiment 9). When mixed with the virus and introduced with it into the skin they did not suppress its activity

unless they were alive, as shown by Experiments 1 and 2. When they or the clasmatocytes of Experiments 3 and 4 had been killed by heating at 53°C.,—a treatment which does not prevent virus fixation (3),—large lesions resulted. For all these reasons it seems unlikely that the observed suppression was incidental to cell necrosis.

The antiviral principle circulating in animals recovered from vaccinia may be carried through washings by blood leukocytes, and retains under such circumstances the neutralizing capacity (8). It has seemed possible that the marked influence of exudate clasmatocytes to suppress the virus, noted in the present work, might have been due to some such happening; but the outcome of Experiment 4 is against this conception, the sera of the animal furnishing the exudate which neutralized the virus being devoid of any significant power of the sort. Furthermore, in Experiment 3 only the clasmatocytes suppressed the virus and this to no unusual extent, although both the clasmatocytic and the polymorphonuclear exudates, and the blood leukocytes as well, were derived from rabbits recently recovered from vaccinia, and hence undoubtedly possessed of potent circulating antibodies. But it should be remarked that the clasmatocytes had been washed by a less searching method than was employed in the case of the other cells.

The antiviral activity of clasmatocytes falls in with Gay's observations on the effect of these cells to combat bacteria (9). Ledingham's histological studies convinced him that "comminution of the reticulo-endothelial cells is the cardinal symptom of virus attack," the surviving elements of this sort rendering the virus inert. Granting, as our facts warrant, that the deterring influence of the reticulo-endothelial cells upon vaccinia resulted from some vital activity on their part, one still cannot conclude that it was the manifestation of a defense mechanism. Local changes incidental to the life of the cells implanted in the intradermal situation may merely have rendered the milieu unfavorable to the virus. But the extent of their influence, as indicated by the space about them in which no virus lesions developed, suggests that more was involved than this.

Our findings with polymorphonuclear cells, both those of exudates and of the blood, corroborate those of Sabin (8), who could obtain no evidence that blood leukocytes exert a destructive effect on vaccinia over and above that due to immune principles derived from the plasma

but carried by them. The observed slight reduction in size of the dermal lesions when blood leukocytes were mixed with the virus, may have been due to some fixation of the latter upon the cells.

The cultivation tests yielded results diametrically opposed to those just considered. The virus underwent a great increase during incubation for 6 and 7 days in cultures containing a large number of Kupffer cells (Experiments 5 and 6), even though most of the fluid medium was replaced daily. This cannot have been because the cells were in poor state,—for they flourished, and rapidly segregated neutral red when tested therewith at the end of the cultivation period; nor was it due to a "blocking" of them with iron,—of which many contained very little. Furthermore, it cannot be attributed to a situation of the virus out of reach of the cells. True, some free virus was present in the cultures when these were first set up, in addition to such as became fixed upon the cells; but this free portion lessened to the point of disappearance during the first few days, as was to have been expected from the combined effects of incubating it at 37°C. and diluting it by replacement of the fluid medium. That the later presence of free virus, in greater and greater amount, was due to an increase in that portion of it which had originally become associated with the Kupffer cells is evident from the large lesions resulting from the inoculation of these cells when the cultivations were discontinued. The findings obtained in this way were wholly against any destructive phagocytosis of the virus by the cells.

The cultivations were kept up long enough for antiviral principles to have appeared in the culture fluid, had forming them been one of the cell activities under the circumstances of the experiments; yet no evidence of any such activity was obtained. It might be urged that so much virus had become fixed upon the cells in the beginning that they had been overpowered. But their great number in the crowded cultures and their excellent first and last state are against this assumption.

Our negative findings as concerns the production of antibodies *in vitro* by Kupffer cells were paralleled by the outcome of numerous experiments made to determine whether these elements will form hemolysins and hemagglutinins. Washed Kupffer cells procured by the magnet method from rabbits and dogs were employed, in some cases from normal animals and in others from animals highly immun-

ized against the test objects (dog, rabbit, rat, and goat corpuscles). Most of the tests were made with fluids drawn off after 24 to 48 hours cultivation of the Kupffer cells, but in some instances the red corpuscles were introduced directly into the cultures. It will suffice to say that the results with these were wholly negative, the failure to demonstrate a formation of hemolysins or hemagglutinins being complete, though the cultured cells fared well to all appearance. In their natural situation within the liver Kupffer cells often manifest a prodigious activity in the phagocytosis of strange red corpuscles, and we had no difficulty in demonstrating that they retained this activity even when containing many iron particles. It was only necessary to run a suspension of rabbit corpuscles into the washed liver of a dog that had some days previously been injected with ferro-magnetic iron, clamp the hepatic vessels, let the organ remain for half an hour at body temperature, and then forcibly flush it out by the usual combination of Tyrode and massage. Numerous Kupffer cells were obtained in this way which held iron particles and in addition had stuffed themselves with the foreign corpuscles. Yet when rabbit corpuscles were introduced into cultures containing great hosts of dog Kupffer cells living on lens paper in fresh normal dog serum, to all practical intents and purposes no phagocytosis occurred. The corpuscles fell past the host of Kupffer cells to the bottom of the dish and even here, where many of them were slowly crawling about, there was no demonstrable phagocytosis. Nor were hemagglutinins formed during the next few days of cultivation in the presence of the strange corpuscles (which gradually broke down).

Extensive tests were made to determine whether Kupffer cells would reactivate normal, homologous serum heated to 56°C. for 30 minutes. The cells do well when cultivated with this as the medium, and it was left on for 24 to 48 hours. The fluid removed from one set of cultures after 24 hours, and centrifuged free from all cells, was found to activate a heated hemolytic serum procured from a rabbit immunized with guinea pig erythrocytes, while furthermore it lost this property on renewed heating to 56°C. for 30 minutes. In control tests with normal, inactivated serum such as had been put on the cultures, as well as with portions of it that had been incubated 24 hours in cell-free culture dishes containing lens paper, no activation took place.

In a succeeding experiment some of the heat-inactivated immune serum employed for the test just described was utilized as the medium of Kupffer cell cultivation, and when it was recovered after 24 hours and centrifuged it was found to hemolyze guinea pig cells without any addition of alexin, and to be inactivated when heated again at 56°C. for 30 minutes. Similar results were obtained in one other such experiment, though the reactivation was less pronounced. In a large, subsequent series of later cultivations, however, with other cells and other specimens of immune rabbit serum these results could not be duplicated; and wholly negative findings were obtained when heated anti-goat rabbit serum of very high titre was utilized in similar experiments. In some final tests cultures were made of Kupffer cells in the inactivated anti-goat serum, and after 24 hours of incubation goat corpuscles were directly introduced into the dishes. They underwent gross agglutination, as was to have been expected, but were not phagocyted or hemolyzed during several hours incubation.

Does the negative outcome of the cultivation experiments with vaccinia, and of nearly all of those with erythrocytes, mean that the Kupffer cells play no rôle in the production of antiviral substances, alexin, hemolysins, hemopsonins, and hemagglutinins? It seems far more likely, in view of the accumulated facts indicating an important rôle for these elements in some of the relations mentioned, that when they are maintained *in vitro* with serum as their culture medium and sole resource, they are unable to carry out tasks of which they are capable under the ordinary circumstances of their life. Parker (10) has recently found that the splenic tissue of an animal injected intravenously some days beforehand with foreign red cells will yield agglutinins for these latter on cultivation *in vitro*, whereas this will not happen if the spleen has been injected with the foreign red cells by way of its artery just prior to explantation. The physiological isolation inevitable to cell cultivation *in vitro*, as at present practiced, is not always an advantage from the experimental point of view. It can very well be a source of misinformation.

McMaster and Kidd (11) have demonstrated that the lymph glands are active in the formation of a neutralizing principle for vaccinia, presumably identical with that which appears in the blood of the animals infected with the virus. Whether the lymphocytes are the cells concerned in its formation has not been determined.

SUMMARY

The pathogenic activity of vaccinia virus is in large part suppressed when it is mixed with living Kupffer cells or clasmatocytes in the test-tube and injected intradermally.

Vaccinia increases in quantity when introduced into cultures of Kupffer cells *in vitro*, and survives in immediate association with these elements. No antiviral principle is elaborated by them under such conditions.

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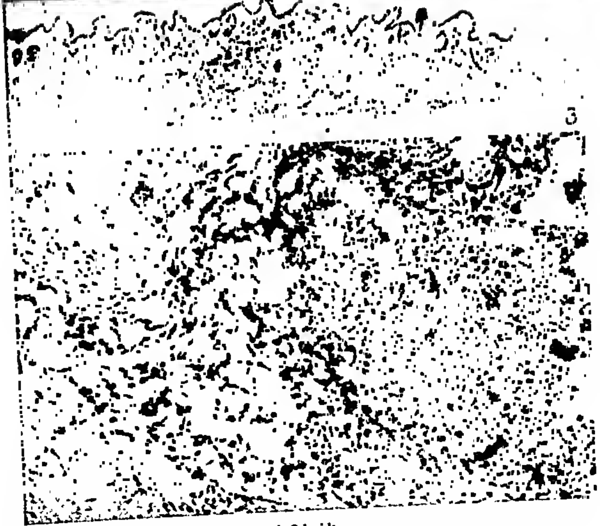
EXPLANATION OF PLATE 39

FIGS. 1 and 2. Vaccinia lesions photographed 5 days after the injection of the materials of Experiment 4.

Virus + (A) Tyrode, (B) fresh serum, (C) heated serum.
" + (D) clasmatocytes; (E) heated clasmatocytes, (F) clasmatocytes and heated serum, (G) heated clasmatocytes and heated serum.
" + (H) aleuronat, (I) heated aleuronat, (J) aleuronat and heated serum.
" + (K) polymorphonuclears, (L) heated polymorphonuclears, (M) polymorphonuclears and heated serum, (N) heated polymorphonuclears and heated serum. × 1/2.

FIGS. 3 and 4. To illustrate the survival of iron-containing Kupffer cells after intradermal injection into a different rabbit: specimens procured 48 hours after the injection. Most of the Kupffer cells contain so much iron as to appear black. It will be seen that they lie scattered to a considerable distance from the immediate site of injection, which is marked by detritus (arrow), and that many have flattened out against the connective tissue fibrils. × 22 and × 153.



Photographed by Joseph B Haulenbeek and Louis Schmidt

(Beard and Rous: Vaccinia virus cultivation with Kupffer cells)

BLEEDING TENDENCY AND PROTHROMBIN DEFICIENCY IN BILIARY FISTULA DOGS: EFFECT OF FEEDING BILE AND VITAMIN K*

By H. P. SMITH, M.D., E. D. WARNER, M.D., K. M. BRINKHOUS, M.D., and W. H. SEEGERS, Ph.D.

(From the Department of Pathology, State University of Iowa College of Medicine, Iowa City)

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Hawkins and Whipple (1) recently described a number of abnormalities which occur commonly in dogs having long standing biliary fistulae. They observed a bleeding tendency which could be cured by feeding bile. If the animals were fed bile periodically in the earlier stages of the experiment the bleeding tendency did not develop. Subsequently, one of us in conjunction with Hawkins (2) showed that the bleeding was due to profound lowering of the plasma prothrombin level. When the bleeding was prevented by feeding bile, the prothrombin remained within normal limits.

Several years ago a hemorrhagic deficiency disease of chicks was described in several laboratories. Dam (3) and Almquist and Stokstad (4) have identified the deficiency factor as a new fat-soluble vitamin, which has been designated the antihemorrhagic vitamin, or vitamin K. Both Schønheyder (5) and Quick (6) have concluded that the bleeding in this hemorrhagic chick disease is due to a low plasma prothrombin. These findings suggested that the biliary fistula dogs also suffer from vitamin K deficiency, a result of faulty absorption when bile is excluded from the gut. That this explanation is correct is indicated by a preliminary report of Greaves and Schmidt (7) on rats. Important confirmation is also supplied by our successful treatment of jaundiced human bleeders with vitamin K (8). A preliminary report of our work with patients was followed almost at once by a similar report from the Mayo Clinic (9).

* Aided by a grant from the John and Mary R. Markle Foundation.

Our animal work includes data on 36 fistula dogs studied during the last 3 years. Though a number of perplexing problems still exist, our experience is in general agreement with the work of Greaves and Schmidt, and in view of their preliminary publication we are presenting at this time a group of our experiments.

Methods

The gall bladder-renal type of biliary fistula described by Kapsinow, Engle, and Harvey (10) was used. The dogs used in these experiments were in good physical condition and free of jaundice.

The kennel diet consisted of mixed table scraps. It contained bread, potato, and vegetables in large quantities, with small amounts of meat and salad dressing. This diet was given unless otherwise indicated. Diet 9 was used in some experiments. It consisted of the following: 1,800 gm. dry white bread, 400 gm. cane sugar, 220 gm. corn starch, 400 gm. canned lean meat, 20 gm. McCollum's salt mixture, and 4,500 gm. water; this mixture was cooked 45 minutes in the autoclave at 15 pounds pressure; then 100 gm. cod liver oil and 100 gm. powdered yeast were added. Diet 15 consisted of dog chow.[1] The restricted vitamin diet (diet 31) consisted of diet 9 without addition of cod liver oil or yeast. In each experiment sufficient food was given so that the animal would maintain its weight. The plasma prothrombin was determined by the method of Warner, Brinkhous, and Smith (11).

Two preparations of vitamin K were used. The first was an extract of dry alfalfa meal. The solvent used in making the extract was a special low boiling gasoline (Skellysolve A). The dry alfalfa meal was placed in flasks and extracted in series, after which the solution was filtered and concentrated by distillation. The second preparation was a potent vitamin K concentrate, partially purified by a modification of the method of Almquist (12). His procedure was followed with these exceptions: (*a*) the initial adsorption steps were omitted, and (*b*) vitamin K was taken up from the final methyl alcohol solution into Skellysolve A by addition of a large volume of water. 1 cc. of the final solution of this concentrate was the equivalent of 100 gm. alfalfa meal. Prior to using either of these preparations, the solvent was evaporated rapidly in a current of air.

Low Plasma Prothrombin Level and the Bleeding Tendency: Effect of Blood Transfusions and Whole Bile Feeding

In biliary fistula dogs the plasma prothrombin level occasionally, though rarely, falls steadily in a very few weeks to the bleeding level. More commonly there is a latent period of several weeks before any

[1] Purina Mills, St. Louis.

significant fall is noticed. Sometimes this latent period is extended over many months. Dog 1 is intermediate in this respect.

Dog 1. (See Chart 1.) Mongrel female, 10.5 kilos. Biliary fistula operation Feb. 19, 1935. Bleeding time and clotting time normal for several months, but on the 214th day the bleeding time was 10 minutes, and on the 230th day the clotting time was 18 minutes. No spontaneous bleeding, however.

On the 304th day a silk stitch abscess appeared and was drained 2 days later. Considerable oozing of blood followed. Transfusion (210 cc.) failed to control the hemorrhage. The incision was infiltrated with thrombin solution and was packed with gauze, after which the bleeding ceased. During the following week gross hematuria and melena were noted. The clotting time on the 310th day was 8 minutes; the bleeding time 3 minutes.

No further bleeding until the 394th day, when moderately profuse vaginal bleeding was noted for several days. No evidence of estrum. On the 422nd day bleeding persisted several hours from a vena puncture site. Transfusion (130 cc.) given; bleeding ceased. Diet now changed from table scraps to a high protein diet, but the dog ate poorly and lost 2.6 kilos in the next 6 weeks. During this period prolonged bleeding followed vena puncture on several occasions, controlled in each case by transfusion.

On the 466th day diet changed to table scraps with which were mixed 50 cc. ox bile daily. Diet continued 7 months and was well eaten. Weight 9.6 kilos. No bleeding since bile feeding was begun. Bile feeding discontinued on the 674th day. For the next 3 months dog was fed table scraps supplemented with canned tomatoes and 4 cc. cod liver oil daily. Weight increased to 11.5 kilos during this period.

Dog 1 is a case in which the latent period was about 12 weeks. Chart 1 shows that the prothrombin level fell to 25 per cent of normal during the next 4 weeks, and to about the 10 per cent level 13 weeks after this. During this entire period of 6 months the dog showed no evidence whatever of spontaneous bleeding.

It has been our experience that bleeding rarely occurs before the plasma prothrombin reaches the 10 per cent level. This bears out our previous experiments (11) showing that the prothrombin level normally is vastly in excess of minimal requirements. Even at the 10 per cent level actual bleeding is conditioned by minor injuries. This dog remained in the danger zone for 4 months without bleeding, but on the 306th day operative drainage of a stitch abscess provided a bleeding site, and hemorrhage was controlled with difficulty. For the next 3 months no bleeding was observed, despite prothrombin levels

of 10 per cent or less. During the next 10 weeks (394th to 466th day) there were 5 episodes of bleeding, once spontaneously from the vagina, and 4 times following vena puncture.

The value of blood transfusions in controlling hemorrhage in such dogs has been shown by Hawkins and Whipple (1). This is well illustrated in this particular dog. As shown on the chart, there was only a slight concomitant rise in the plasma prothrombin. This rise, however, was sufficient to bring the prothrombin above the bleeding

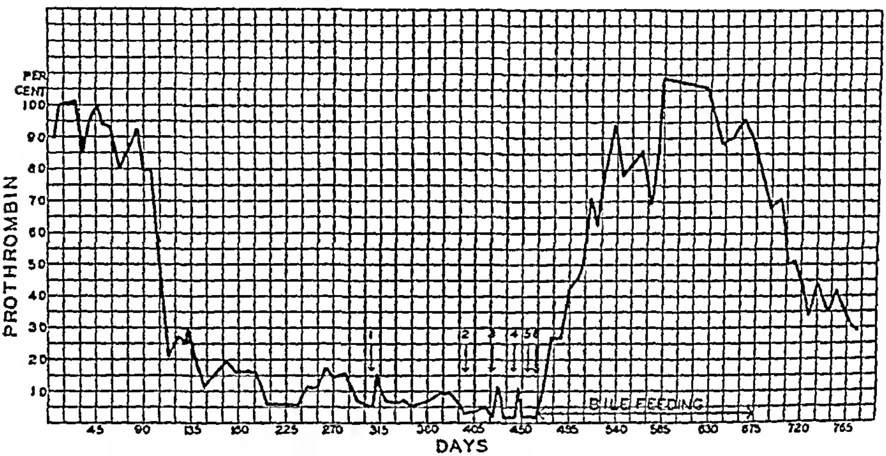

CHART 1. Dog 1. Arrows indicate occasions when prolonged bleeding occurred; (1) bleeding from operative site (stitch abscess), transfusion 210 cc.; (2) vaginal bleeding; (3) bleeding from vena puncture site, transfusion 130 cc.; (4) bleeding from vena puncture site, transfusion 110 cc.; (5) bleeding from vena puncture site, transfusion 160 cc.; (6) bleeding from vena puncture site, transfusion 75 cc.

level. The increased level could not be maintained, however, and in a short time bleeding recurred. The rapidity with which the prothrombin again fell to the extremely low levels gives an indication as to the rate of prothrombin utilization in these animals.

Beginning on the 466th day, 50 cc. ox bile were added daily to the diet of this dog. From this point on the prothrombin level rose steadily, reaching normal levels 4 months later. For the next 3 months, continued bile feeding maintained the prothrombin at an essentially normal level. On discontinuing bile feeding the prothrombin level fell again, reaching the 29 per cent level in 14 weeks. It is

worthy of note that no latentperiod preceded this fall. One might postulate that the reserves concerned in prothrombin manufacture were not as great as originally.

Effect of Bile Salt Feeding on the Prothrombin Level

Dog 2 showed a dangerously low prothrombin level in about 4 months after operation (Table I). Beginning on the 156th day of the experiment, bile salt was added daily to the diet over a period of approximately 2 months. The dry salt was thoroughly mixed with the food, which was promptly eaten. Within 10 days a noticeable rise in plasma prothrombin had occurred, and in 24 days it had reached the 72 per cent level. This level was maintained for the next 3 weeks after which a small decline was evident. The failure to reach normal levels occurred despite an increase in the amount of bile salt added to the diet.

TABLE I

Effect of Bile Salt Feeding on the Prothrombin Level

Dog 2; gall bladder-renal fistula, Feb. 20, 1935; weight 18 kg.

Day of experiment	Bile salt feeding daily	Prothrombin*
	gm.	*per cent*
0	0	110
84	0	61
106	0	24
126	0	8
155	0	7
156–158	1.0	7
159–165	2.5	17
166–172	2.5	34
173–179	2.5	72
180–186	2.5	70
187–193	2.5	78
194–200	4.0	70
201–206	4.0	57
207–211	4.0	51
212–218	0	51

* Prothrombin determinations at end of each period.

Effect on the Prothrombin Level of Feeding Antihemorrhagic Vitamin Preparations

Chart 2 shows the effect of feeding the antihemorrhagic vitamin to chronic biliary fistula dogs. In each case a number of months had elapsed since operation and gradually the plasma prothrombin level had fallen to 37 per cent or less. In no case had the diet been enriched in regard to the antihemorrhagic vitamin. In dogs 4 and 5

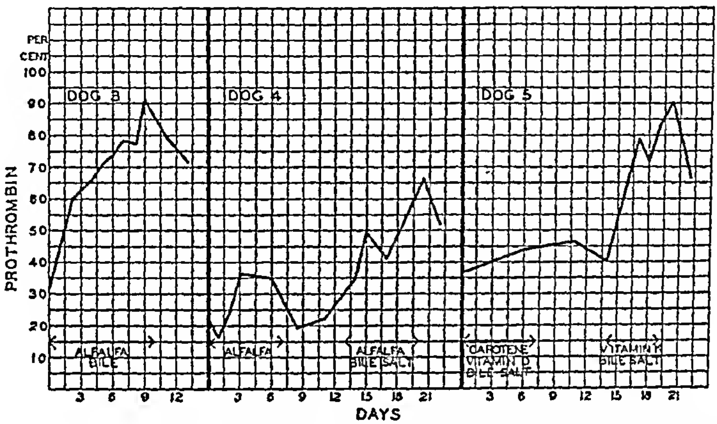

CHART 2. Dog 3, female, 10 kilos. Fistula operation 29 weeks previously. Table scrap diet throughout. Alfalfa extract (150 gm. equivalent) + 75 cc. ox bile mixed with food daily for 10 days, as shown above.

Dog 4, female, 12 kilos. Fistula operation 27 weeks previously. Diet 15 for last 16 weeks. Supplement of alfalfa extract (200 gm. equivalent) daily for 7 days; for second 7 day period, supplement of alfalfa (200 gm. equivalent) + 0.6 gm. bile salt daily, shown above.

Dog 5, female, 9 kilos. Fistula operation 57 weeks previously. Diet 31 for last 15 weeks. Carotene (5 mg.) + vitamin D (drisdol, 15,000 U. S. P. units) + 0.6 gm. bile salt daily for 7 days. Vitamin K concentrate (500 gm. alfalfa equivalent) + 0.6 gm. bile salt daily during second 7 day period.

standard diets were used which are low in the known sources of the vitamin. Dog 3, on a variable diet of table scraps, followed approximately the same downhill course.

Dog 3, Chart 2, shows the response to appropriate dietary supplements. A daily supplement of vitamin extracted from 150 gm. alfalfa meal was given emulsified in ox bile and mixed with the food. The response was immediate. Within 2 days the plasma prothrombin